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100

Williams, Carroll M.
Ecdysone and ecdysone-analogues: Their assay and action on diapausing pupae of the cynthia silkworm 344
Zimmerman, Selma B., Tetuhide H. Murakami and Arthur M. Zimmerman
The effects of selected chemical agents on furrow induction in the eggs of Arbacia punctulata 356

No. 3. June, 1968

Ahearn, Gregory A.
A comparative study of P^{32} uptake by whole animals and isolated body regions of the sea cucumber Holothuria atra 367
Henley, Catherine
Refractile bodies in the developing and mature spermatozoa of Childia groenlandica (Turbellaria: Acoela) and their possible significance 382
Björnberg, T. K. S., and Karl M. Wilbur
Copepod phototaxis and vertical migration influenced by xanthene dyes .. 398
Halldal, Per
Photosynthetic capacities and photosynthetic action spectra of endozoic algae of the massive coral Favia 411
Ishihara, K.
Chemical analysis of glycoproteins in the egg surface of the sea urchin Arbacia punctulata 425
Kriebel, Mahlon E.
Studies on cardiovascular physiology of tunicates 434
Lochhead, John H.
The feeding and swimming of Conchoecia (Crustacea, Ostracoda) 456
Macklin, Martin
Reversal of cell layers in hydra: A critical re-appraisal 465
Mun, A. M., and Jane Longo
Growth stimulation of embryonic spleen graft by adult chicken spleen across a membrane filter barrier 473
Paulson, Theodora C., and Rudolf S. Scheltema
Selective feeding on algal cells by the veliger larvae of Nassarius obsoletus (Gastropoda, Prosobranchia) 481
Sexton, Owen J., and Eckhard H. Hess
A pheromone-like dispersant affecting the local distribution of the European house cricket, Acheta domestica 490
Simon, Joseph L.
Occurrence of pelagic larvae in Spio setosa Verrill, 1873 (Polychaeta: Spionidae) 503
Yoshida, M., and H. Ohtsuki
The phototactic behavior of the starfish, Asterias amurensis Lütken 513

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EFFECTS OF SALINITY AND NUTRIENTS ON MONO- AND DIAXENIC CULTURES OF TWO STRAINS OF ARTEMIA SALINA[1]

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Bacteria-free cultures of crustaceans are needed to determine the nutritional value of different algae and to study the requirements of typical grazers.

Previous work was done with the bisexual Utah strain of *Artemia salina* (Great Salt Lake) (Provasoli *et al.,* 1959b). Wintering eggs were commercially available, easily washed free of bacteria, and easy to hatch. These obvious advantages facilitated development of monaxenic and axenic culture methods (Provasoli *et al.,* 1959a). However, the Utah strain had disadvantages: to obtain successive generations males and females had to be brought together to copulate, but copulation was not always successful and in most cases, wintering eggs were deposited. These eggs, for hatching, had to be collected, air-dried, and reincubated in a hatching medium. These aseptic manipulations enhanced risk of contamination. Detection of possible long-range nutritional deficiencies, which often appear only after several generations, would be too laborious. To by-pass these difficulties the parthenogenetic strain of *Artemia salina* from Comacchio was axenized.

MATERIALS AND METHODS

Source

The Comacchio strain of *Artemia* was given to us by Dr. C. Barigozzi of the Dept. of Genetics, University of Milano. His cultures had been established from an adult female obtained from wintering eggs, collected at the Comacchio salt ponds near Venice, and were maintained in Flemming's synthetic sea water with *Dunaliella tertiolecta* and Fleischmann yeast as food (Ballardin and Metalli, 1963). Under optimal agnotobiotic conditions adult females released a new brood of viviparous nauplii every four days.

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Dr. Barigozzi also sent a few wintering eggs; occasionally this viviparous strain of *Artemia salina* deposits durable eggs.

Axenization trials

1. Wintering eggs

The techniques of Gibor (1956a) and Provasoli *et al.* (1959b) needed minor modifications to wash free of contaminants the wintering eggs of the many strains of *Artemia.*

Unfortunately, the available wintering eggs of the Comacchio strain were so few that we could not wash them by the usual flotation method. These eggs were washed by serial transfers in sterile Pyrex spot-test slides: 9 baths (1 ml.) in sterile sea water followed by 3 baths in 1:1000 Merthiolate solution (lasting a total of 10 minutes) and 3 baths in sea water. They then were allowed to hatch; each hatched nauplius was in turn also washed repeatedly. In spite of the added precautions all nauplii were bacterized. The Comacchio eggs, unlike those of other strains, were heavily coated with detritus and, most likely, bacteria were not dislodged by our wash method.

2. Nauplii

Extensive serial washing of the viviparous nauplii collected from crude cultures in our laboratory failed to eliminate the bacteria, probably because the gut microflora remained untouched. With the hope that ready-to-hatch nauplii still inside the ovisac might be aseptic, a female with an ovisac was passed through several baths of Merthiolate 1:1000 solution and 75% alcohol. The artemia died, and the ovisac was opened in sterile sea water with glass needles under a germicidal hood. None of the nauplii recovered alive were bacteria-free.

3. Antibiotic tolerance

Because of the previous failures it became desirable to determine the tolerance of the adults and the nauplii to various antibiotics. Both stages had similar tolerances (Table I).

The sensitivity of the bacterial flora was tested with sensitivity discs (Difco). No single antibiotic completely inhibited bacterial growth. Several mixtures of the most effective antibiotics were tried (Table II).

Purification technique

Antibiotic mix 2 (Table II) in the wash solutions permitted elimination of all bacteria, but a *Saccharomyces* persisted. Fungicides could not be used because they were more toxic to *Artemia* than to the yeast. The yeast was ultimately eliminated by growing the *Artemia* in high-salinity media; details follow.

Elimination of bacteria. Synthetic salt solutions of different salinities were made from appropriate dilution of the stock salt solution mix MGSL (Table III). Unless otherwise indicated, growth media were also prepared from this stock salt mixture.

TABLE I

Tolerance to antibiotics of Artemia adults and nauplii
(μg./ml.)

Antibiotics	Safe(1)	Slight(2) inhibition	Marked(3) inhibition	Toxic(4)
Penicillin*	1850	3300	9900	
Streptomycin	200	300	400	1000
Polymixin*	228	338	435	456
Chloramphenicol	25	50	56	224 (Ab)
Naramycin	10	15	25	50
Kanamycin	250	500	750	
Neomycin	5	10	15	20 (Ab)
Nalidixin	50	100	150	200
Tetracycline	5	7.5	9	15 (Ab)
Trichomycin		50	75	100 (Ab)
Fungicides:				
Candicidin	2.5	3	5	7.5
Nystatin	10	15	30	
5,6-Dichlorobenzoxozaline**				0.1 ml.***
Lauryl hydroxamate**				0.1 ml.***
Methenamine mandelate	500	1000	2000	3000

* Units/ml.: pencillin 1 mg. = 1650 units; Polymixin 1 mg. = 7760 units.

** Obtained from Geigy Co.

*** ml. of saturated solution/ml. of medium.

Ab = Algae bleach within 24 hours.

(1) No apparent effect to *Artemia* adults and nauplii after 37 days.

(2) Slight inhibition of growth; animals recovered readily if transferred to *no* antibiotics after first signs of inhibition (7–30 days).

(3) Marked inhibition of growth; generally adults did not recover, nauplii often recovered but did not reach adulthood.

(4) Death occurs in 1–24 hours. Nauplii seemed more sensitive.

TABLE II

Antibiotic mixes
(μg./ml.)

Antibiotics and fungicides	Mix 2** 1 ml. =	Mix 2M** 1 ml. =	Mix 3*** 1 ml. =
Penicillin*	16500	16500	
Streptomycin	1000	1000	
Polymixin*	2880	2880	
Chloramphenicol	250	250	250
Candicidin		25	
Methenamine mandelate		1000	
Trichomycin			1000
Tetracycline			100
Neomycin			100

* Units/ml.: penicillin 1 mg. = 1650 units; Polymixin 1 mg. = 7760 units.

** Used at 0.1 ml./ml. for adult *Artemia*; 0.05 ml./ml. for nauplii.

*** Used at 0.1 ml./ml. to wash free of algae nauplii intended for nutritional experiments. *Artemia* nauplii must not be exposed to this mixture any longer than 6 hours; the nauplii were more sensitive than later stages.

A female with eggs in the ovisac was transferred to 30 ml. of 3 g.% salt solution to which was added 0.1 ml./ml. of antibiotic mix 2 and *D. tertiolecta* as the food organism. The female was transferred every hour during the working day to similarly prepared fresh media. This effectively prevented accumulation of fecal matter and bacteria. On the second or third day each nauplius as it emerged from the ovisac, was washed through 10 successive baths (30 minutes stay in each) of 10 ml. of the same salt-solution but with only 0.05 ml./ml. of antibiotic mix 2 and no food organisms. The nauplii do not feed for the first few hours. After the last wash *D. tertiolecta* was added; 24 hours later the nauplii were washed free of antibiotics and food organisms, and transferred into STP (Provasoli *et al.*, 1959a) for $\frac{1}{2}$ hour to test for sterility. They were then placed into the maintenance medium MSWE or in MGSL (at 3% total salts) enriched with algal nutrients, and food organisms added. Ten larvae so washed proved bacteria-free. A *Saccharomyces* was detected a month or so later (STP is not a particularly good medium for yeasts).

TABLE III

*MGSL stock salt solution**

g.% (*w/v*)

NaCl	17.30	
KCl	0.64	
$MgSO_4 \cdot 7H_2O$	3.40	
$MgCl_2 \cdot 6H_2O$	1.91	
$CaCl_2$	0.09	
$Na_2CO_3 \cdot H_2O$	0.03	
	23.37	
	2.76	H_2O of hydration
	20.61	g.% total salts less H_2O of hydration.

Dilutions (v/v) of this stock salt solution may be autoclaved without precipation.

The pH after autoclaving fluctuated between 7.0 and 7.8, well within the tolerance of the *Artemia* and the algae.

* MGSLE = MGSL + E enrichment (Table IV, note 2).

MGSLG = 50 mg.% glycylglycine are added as pH buffer.

Elimination of the yeast. Since, as noted, artemia nauplii and adults proved extremely sensitive to fungicides (Table I), we exploited the adaptability of the crustacean to high salinities. Surprisingly, the adult female could not tolerate sudden shifts in salinity. Gravid females when transferred from a 3% to a 6% salt solution invariably dropped the immature eggs and soon died. Nauplii, on the contrary, survived sudden salinity shifts: they could be transferred directly from a 3% to any concentration in the range 0.5–20.5% salt solution. Survival of the nauplii varied with age: 80% of the 6-hour-old nauplii survived direct transfer into 0.5–9% salts, and 35% when transferred into 12–20.5% salts; after the first naupliar molts, sensitivity to salinity changes increased and percentage survival decreased.

Use of a wide range of salinities (0.5–20.5%) entailed change in food organism:

D. tertiolecta did not grow at salt concentrations > 6%. Several algae were tested for ability to grow at the full range of concentrations after adaptation by serial transfer in gradually more diluted or concentrated solutions until the algae grew almost equally well at all concentrations.

Stephanoptera gracilis did not grow well < 6%. *Dunaliella parva, D. salina,* and *D. viridis* grew at all salinities, but *D. parva* bleached readily; *D. viridis* was acceptable to adult *Artemia* and not to the nauplii, while *D. salina* was acceptable to the nauplii as well as the adults, but grew slowly and was grazed too rapidly by the developing *Artemia.* Therefore, a mixture of *D. salina* and *D. viridis* was used.

Viviparous nauplii produced in MSWE (Provasoli *et al.*, 1959b, p. 254) by a bacteria-free but yeast-contaminated Comacchio female were transferred and grown to adulthood at the different salinities. Best growth was obtained in the 9% media. Viviparous nauplii released at this salinity were washed 10 times in 10 ml. of 9% media containing 0.5 ml./10 ml. of antibiotic mix 2M. Several nauplii proved free of bacteria and yeast. These were used to start the stock line which has been kept for over 3 years.

MAINTENANCE OF STOCK CULTURES

Several cumbersome trials led to the following two simple procedures for maintaining axenic cultures.

(1) Equal quantities (0.5 ml.) of a full-grown culture of *D. viridis* and *D. salina* were inoculated in screw-capped tubes (20 × 125 mm. Pyrex) containing 10 ml. of an autoclaved sea water enriched with organics (MSWE medium). After 10–15 days growth at 20–26° C. and *ca.* 200 ft. c., the algae cultures were very dense. One washed, newborn nauplius was inoculated in each of 10 tubes. Twenty to 25 days later, 3–5 of the 10 nauplii had become fertile females. The nauplii released were collected and the process repeated to carry on the new generations; any extra nauplii were used to carry on other experiments.

(2) The method was further simplified: 0.5 ml. of a dense suspension of the 2 algae and three washed new-born nauplii were inoculated simultaneously into each of a set of 5 tubes of STP. After 8–10 days at 20–26° C. and 200 ft. c., one of the three nauplii in each tube invariably outgrew the others. The two slow-growing artemia were removed, the other was left in the tube. Within 10 more days it developed into a fertile female; 5–6 days later the first batch of viviparous nauplii was released.

The nauplii taken from the parent's tube were washed three times in 10 ml. each of STP, the third wash tube was incubated in the dark, serving as a sterility test. Most of the algae were eliminated in the first two washes; therefore in the third tube the bacteria—if present—became evident (turbidity) before the algae masked them.

Several algae which had proved to be excellent food organisms for the Utah strain were also tested with the Comacchio strain, in the hope that some of them might be better for maintaining the bacteria-free stocks.

The algae were inoculated into STP, allowed to grow for 15 days, and then the nauplii were added. *Phaeodactylum tricornutum,* like *Dunaliella viridis,* was not acceptable to nauplii and early metanauplii. The diatom may have been too big to be ingested or it may have been indigestible. However, it was an excellent food organism for the older and larger metanauplii and the adults. *Nitzschia acicularis* grew abundantly but growth of the artemia was slow (from nauplii to adults 40–50 days). A marine *Chlorella* sp. (No. 580, Guillard) allowed better growth—nauplii to adult in 30 days—but it was very difficult to wash the nauplii free from them; antibiotic mix 3 was not effective against it. *Rhodomonas lens* was an excellent food organism (fertile female in 18 to 20 days) but it grew slowly and was quickly and completely grazed: suspensions of the algae had to be added to the culture tubes every few days—an impractical procedure. *Isochrysis galbana* was also a good food organism but it was too sensitive to laboratory temperatures (20–28° C.).

Dunaliella salina + *D. viridis* remained best; *D. viridis* + *P. tricornutum* was also adequate but such cultures had to be watched closely. If the *P. tricornutum* dominated the system, growth of artemia equalled or bettered that obtained with the combination *D. salina* + *D. viridis*; if *D. viridis* dominated, *P. tricornutum* began to bleach and the *Artemia* died.

TRIALS WITH ARTIFICIAL MEDIA

Selective elimination of algal food. For nutritional experiments in artificial media, the viviparous parthenogenetic animals grown on live food had to be algae-free. Prolonged and tedious serial washes, spanning two or more days, were used at first. Fortunately, during the tolerance tests of the Comacchio strain to the antibiotics, tetracycline, trichomycin and chloramphenicol proved particularly inhibitory to *D. viridis* and *D. salina* and so served to eliminate the algae.

A brood of the Comacchio strain (30–60 nauplii preferably < 6 hours old) was collected by Pasteur pipette and transferred through 10 consecutive washes to sterile sea water + 1 ml./10 of antibiotic mix No. 3 (Table II). The nauplii were left in each wash 20 minutes, the whole operation lasting four hours. This procedure inhibited the algae without injuring the nauplii. If the nauplii had already begun feeding (8 or more hours old) the gut appeared green; in such cases after every 3 baths in sea water, the nauplii were transferred to, and left for 30 minutes, in a tube containing, in addition to the antibiotic mix 3, particulate starch (dry insoluble rice starch, autoclaved, suspended in H_2O, added dropwise). *Ad libitum* ingestion of the particulate starch induced defecation, thus cleaning the gut of surviving *D. viridis.* These manipulations were made in a transfer hood under a germicidal UV lamp.

Growth in artificial media. Several artificial media used to grow the axenic Utah strain of *Artemia* from nauplii to fertile females and egg deposition were tested with the Comacchio strain. The media were composed of (1) a liquid phase containing mineral salts, amino acids, liver extract, hydrolyzed DNA and RNA, eight vitamins, cholesterol, and (2) a fine particulate phase composed of precipitated egg albumin and rice starch. These media proved inadequate for the Comacchio strain. Nauplii (*ca.* 0.9 mm.) developed in 20–30 days into big metanauplii (*ca.* 3.5 mm., ½ the size of an early adult) which did not grow any further and died. If early metanauplii were inoculated in the artificial media they developed into young (5–7 mm.); if young adults were inoculated, they developed into infertile females (7–10 mm.). Young females fed with these artificial media were able to produce 1–2 normal viviparous broods (30–120 viable nauplii) but died soon after. Variations of the components of the media did not substantially improve growth. Evidently the Comacchio strain needed additional growth factors.

Effects of Salinity and Enrichments on the Growth of Artemia

Purification of the Comacchio strain coincided with preliminary experiments on the Utah strain to determine the effects of salinity on egg production (D'Agostino, 1965).

At first the food algae for the Utah strain (a mixture of *D. tertiolecta, D. parva,* and *D. primolecta*) were grown on agar slants, the algae were scraped off, suspended in sterile sea water, centrifuged, and added as slurry *ad libitum* (as they were consumed by the growing *Artemia*) in the various dilutions of MSGL (0.5–20.5% salt concentrations).

Unexpectedly, even though the algae were abundant, only infertile Utah adults were obtained at salt concentrations < 3%; some nauplii were produced at 3%; and durable eggs were obtained only at concentrations > 6% (Table IV). In a later experiment the conditions were fortuitously changed: (a) the MGSL base was enriched with E (Table IV, note 2) to favor algal growth; (b) having

Table IV

Effect of enrichments and salinities on A. salina

Artemia strain % Total salts	Utah Strain (Great Salt Lake)						Comacchio Strain (Italy)					
	Inorganic enrich. (1)			Organic enrich. (2)			Inorganic enrich. (3)			Organic enrich. (4)		
	Stage reached	Nauplii (5)	Eggs (6)	Stage reached	Nauplii	Eggs	Stage reached	Nauplii	Eggs	Stage reached	Nauplii	Eggs
0.5	Y-♀	0	0	♀	0.3	6.67	J	0	0	♀	5.0	0
1	♀	0	0	♀	1.3	6.8	Y	0	0	♀	5.4	0
3	♀	2.5	0	♀	0.8	4.73	♀	0	0	♀	0.2	0
6	♀	3.6	2.4	♀	1.8	11.20	♀	4.8	1.0	♀	1.9	0
9	♀	4.0	2.1	♀	3.0	5.69	♀	4.3	4.0	♀	6.3	2.9
12	♀	4.6	2.2	♀	1.9	3.63	♀	1.8	2.1	♀	5.8	2.4
15				♀	5.0	4.37	♀	4.7	3.1	♀	6.0	0.9
18				♀	3.1	2.2	♀	4.9	3.2	♀	5.3	2.1
20.5				♀	1.3	0.66	♀	7.9	2.3	♀	3.0	1.7

(1) Base = MGSL dilutions, no enrichment; various species of *Dunaliella*, grown on inorganic agar slants added *ad libitum*. One experiment.

(2) Base = dilutions of MGSL enriched with 5 ml./100 of STP (see text); or with 5 ml./100 STP + 2.5 ml./100 ES; or with 1 ml./100 E + 5 ml./100 STP. One ml./100 E introduces in percentage final medium; $NaNO_3$ 30 mg.; Na_2 glycerophosphate 5 mg.; UII metals 0.1 ml.; vitamins 8a 0.1 ml.; B_{12} 0.1 μg.; Fe (as EDTA) 50 μg. (see notes for Table V). Average of these 3 experiments.

(3) Base = dilutions of MGSLG (see note Table III) enriched with 5 ml./100 of ES or with 2.5 ml./100 ES + 1 ml./100 E_2. Average of these 2 experiments.

(4) Base = dilutions of MGSLGE (see note Table III) enriched with 1 ml./100 of E_1. One experiment.

(5) Viable nauplii produced per female/days of female life.

(6) Durable eggs produced per female/days of female life.

exhausted the supply of algae grown on slants just when most Utah artemias had reached the adult stage, algae grown in STP liquid medium were added unwashed, thus introducing with the algae about 0.5–1 ml. of STP per 10 ml. of base. These changes induced egg deposition at all salt concentrations even in the range 0.5–3% (Table IV), indicating that the organic enrichment affected egg production at low salinities. This effect was similar to that observed with *Tigriopus* (Shiraishi and Provasoli, 1959), the food algae, grown on a medium already containing an organic enrichment (5 ml./100 of STP), became an insufficient food source after several aseptic generations of *Tigriopus*; at the time the nutritional insufficiency was corrected by the addition of a vitamin mixture to the medium in which the algae and *Tigriopus* were growing.

TABLE V

Enrichments
(% in final medium when used at stated addition)

Enrichment designation	STP	E_3	ES	E_2	ES+STP	E_1	STP
Components \ addition	5 ml./100	1 ml./100	5 ml./100	1 ml./100	2.5 ml./100 each	1 ml./100	100%
Soil extract	0.25 ml.				0.125 ml.		5 ml.
$NaNO_3$	1 mg.	5 mg.	17 mg.	5 mg.	9 mg.	25 mg.	20 mg.
K_2HPO_4	0.05 mg.				25 μg.		1 mg.
Na_2 glycerophosphate		2 mg.	2.5 mg.	2 mg.	1.25 mg.	10 mg.	
$Na_2SiO_3 \cdot 9H_2O$						5 mg.	
Metals UII⁽¹⁾				0.01 ml.		0.05 ml.	
Metals PII⁽²⁾			1.25 ml.	0.05 ml.	0.62 ml.	0.12 ml.	
Metals SII⁽³⁾				0.05 ml.		0.12 ml.	
Fe (as EDTA)⁽⁴⁾			0.125 mg.	5 μg.	62 μg.	50 μg.	
Vitamins 8a⁽⁵⁾	0.005 ml.				0.0025 ml.	0.05 ml.	0.1 ml.
Thiamine			25 μg.		12 μg.		
Biotin			0.25 μg.		0.12 μg.		
B_{12}			0.5 μg.		0.25 μg.		
Sucrose (s) or glucose (g)	5 mg. (s)				2.5 mg. (s)	25 mg. (g)	100 mg. (s)
NaH-glutamate	2.5 mg.			5 mg.	1.25 mg.		50 mg.
DL-alanine	0.5 mg.			1 mg.	0.25 mg.		10 mg.
Glycine	0.5 mg.			1 mg.	0.25 mg.		10 mg.
Glycylglycine						25 mg.	
Yeast autolysate (A) or extract (E)	1 mg. (A)				0.5 mg. (A)	0.5 mg. (E)	20 mg. (A)
Trypticase	1 mg.				0.5 mg.		20 mg.
Liver Oxoid L.25						0.5 mg.	
Urea						1 mg.	
Tris buffer			25 mg.		12.5 mg.		

(1) Metals U II (Aldrich & Wilson 1960) 1 ml. contains: Na_2 EDTA 0.15 mg.; $FeCl_2 \cdot 4H_2O$ 1 μg.; $CuCl_2$ 1 μg.; $MnCl_2 \cdot 4H_2O$ 10 μg.; $ZnCl_2$ 5 μg.; $NiCl_2 \cdot 6H_2O$ 1 μg.; $AlCl_3 . 6H_2O$ 5 μg.; $CoCl_2 \cdot 6H_2O$ 5 μg.; RbCl 10 μg.; $BaCl_2$ 1 μg.; H_2SeO_3 5 μg.; NH_4VO_3 1 μg.; TiO_2 5 μg.; $ZrOCl_2 \cdot 8H_2O$ 5 μg.; $K_2Cr_2O_7$ 5 μg.; $SrCl_2 \cdot 6H_2O$ 5 μg.; $Na_2MoO_4 \cdot 2H_2O$ 5 μg.; H_3BO_3 5μg.; CsCl 5 μg.; $(NH_4)_2$ Ce $(NO_3)_6$ 1μg.; $CdCl_2 \cdot 2\frac{1}{2}$ H_2O 1μg.; $SnCl_2 . 2H_2O$ 1μg.; $RuCl_3$ 1 μg.; $RhCl_3$ 1 μg.

(2) (3) (5) See Provasoli *et al.* (1957).

(4) Dissolve 351 mg. of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 330 mg. Na_2EDTA in 500 ml. H_2O; 1 ml. of this solution = 0.1 mg. Fe.

Various enrichments (Table V) were formulated and tried on the Utah and Comacchio strains employing the *Tigriopus* technique, *i.e.*, the algae and the *Artemia* nauplii were inoculated simultaneously in the enriched media. Salinity-adapted *D. salina* and *D. viridis* were employed as food organisms. The effect on egg deposition at low salinities induced by the combination E + STP was confirmed with this technique for the Utah strain with the *D. salina* + *D. viridis* food combination (Table IV).

Similar experiments were done with the Comacchio strain. Table VI shows the effect of different enrichments and different salt concentrations after 26 days growth. *D. salina* was inoculated in duplicate sets of tubes and allowed to grow 10 days; the density of the algal growth was similar in all enrichments and salinities. Single algal-free washed nauplii were inoculated in each tube and the experiment read at the 26th day, when the algae had been grazed almost completely in some tubes while much algae still remained in other tubes. As evident, the effect of low salinities was even more pronounced in the Comacchio strain: concentrations $< 3\%$ slowed development; this effect was gradually overcome as the additions became richer in organic nutrients, E_1 being the most satisfactory enrichment.

Experiments in which the algae were seldom limiting (weekends) were done by transferring the Comacchio artemias to a new tube of algae whenever the previous tube became depleted of algae due to grazing (Table IV, columns 3, 4). Salinities below sea water concentration with inorganic enrichment not only slowed growth but allowed growth only to juvenile or young stages, with no further development even though food was abundant and the animals remained alive for 10–20 days. The organic enrichment E_1 overcame the low-salinity effect and allowed growth to adult fertile viviparous females.

TABLE VI

Effect of salinity and enrichments on the Comacchio strain(1)

Enrichment \ % Total salts	0.5%	(3)	1 g.%	(3)	3 g.%	(3)	6 g.%	(3)
STP 5 ml./100	IV medium(2)	+++	Juvenile	+	IV big	+	IV big	+
E_3 1 ml./100	IV medium	+	Juvenile	+	Young	±	Young	±
E_2 1 ml./100	IV medium	+++	Young	–	Young	–	Young	±
STP 5 ml. + ES 5 ml./100	Young	+++	Young ♀	+	♀	–	♀	++
E_1 1 ml./100	♀	–	♀	+	♀	+	♀	±

(1) 26 days growth; basal medium = dilutions of MGSL.

(2) For description of morphological appearance see Provasoli and Shiraishi (1959b). The length in mm. of the various stages follows: IV medium, 2.5–3.5; IV big, 4–6; juvenile, 6–8; young, 8–9; young female, 9–12; fertile females, 12–14.

(3) Density of algae at 26 days: +++ very many; ++ and + many; ± few; – scarce.

EFFECTS OF SALINITY AND FOOD AVAILABILITY ON OVO- AND VIVIPARITY

The two strains of *Artemia* differ in reproduction. The Utah strain is amphigonic and after copulation may deposit several types of eggs in the laboratory: (a) green or white eggs which fail to hatch; (b) pinkish eggs which may hatch 24–48 hours after deposition; (c) hard-shelled pale or dark-brown eggs equivalent to the wintering (or durable) eggs found in salinas and salt lakes; such eggs when deposited are in diapause and require rehydration for hatching.

The Comacchio strain is parthenogenetic oviparous and viviparous; usually the eggs hatch in the ovisac and the nauplii emerge. The organic enrichment at salt concentrations $\leqq 3\%$ predominately induces wintering eggs in the Utah strain; the Comacchio strain is exclusively viviparous. Interestingly, wintering eggs were produced by the Comacchio strain at salt concentrations $\geqq 9\%$. The results in Table IV are expressed as numbers of nauplii or durable eggs per day of female life and are the mean of several experiments. Most females lived 30 ± 5 days; one lived 10 days and four 37–40 days.

Both strains of *Artemia* grazed the algae more vigorously at higher salinities; therefore the adults had to be transferred more often to new tubes preinoculated with algae to try to keep them fed *ad libitum.* Production of nauplii or wintering eggs varied more widely in higher salinities; this was correlated with periods of less available food, *i.e.*, during weekends. To investigate this possibility, the Comacchio strain was selected because, being parthenogenetic, only one animal—the female—was needed, making it easier to maintain a more constant supply of algal food under aseptic conditions. Mature females were transferred singly in tubes rich in algae. In 6–7 days they produced a viviparous brood of nauplii; by this time the algae had almost completely disappeared. The nauplii were taken out of the tube and the female was starved; 5–7 days later durable eggs were produced. The female was now transferred to a tube rich in algae and in 5–7 days produced again a viviparous brood. This experiment was repeated several times in MGSL base at 5% + E_1 enrichment or in a commercial sea water-type salt mix (RILA, same concentration) + E_2 + STP.

Ballardin and Metalli (1963), working with bacterized cultures fed with a "marine *Chlaymdomonas*" (supplied by us = *Dunaliella tertiolecta*) observed that under their conditions (artificial sea water + organic enrichment) the Comacchio strain tended to deposit wintering eggs. They found no correlation between oviparity and type of food, overcrowding, higher salinities, temperature, and illumination. It is not clear whether they subjected the Comacchio strain to deliberate starvation.

"Hypo-alimentation" of the S. Gilla (Cagliari) strain, from larval stages to the 1st deposition gave viviparous nauplii, but if scarcity of food continued, the 2nd deposition was almost completely oviparous. They concluded that oviparity in the Cagliari strain is influenced by the length of hypo-alimentation. Their culture conditions were different from ours; they achieved hypo-alimentation by less frequent additions of algae, thus causing slower growth and higher mortalities. We starved full-grown and fertile females, only during the egg production-deposition period.

DISCUSSION

Experiments on *Tigriopus* and *Artemia* had shown that the nutritional value of different species of algae varied widely even among species of the same genus: only four algae of the 20 tried were a complete food for at least one generation of both Crustacea. With *Tigriopus* (whose eggs hatch immediately), only one species of algae, *Monochrysis lutheri,* was a complete food for more than 20 generations of the crustacean. Nutritional deficiencies developed after 1–9 generations of *Tigriopus,* depending on the species of algal food, with gradual reduction in size of adults and subsequent infertility. With two species of algae, *Isochrysis* and *Chroomonas,* normal growth and fertility of *Tigriopus* was restored by adding a vitamin mixture or glutathione. At the time, speculations were made as to whether the vitamins were absorbed directly by *Tigriopus* or were taken up by the algae, and perhaps had affected algal metabolism in some way (Shiraishi and Provasoli, 1959).

In the experiments with the two strains of *Artemia* the nutritional deficiencies were most evident when the organisms were subjected to the physiological stress of low salt concentrations. The possibilities are: has the osmotic stress affected mainly *Artemia* (*i.e.*, by changing its nutritional requirements) or mainly the algae (by changing the algal metabolism, hence their nutritional value) or has it affected both—prey and predator? Strangely, as in *Tigriopus*, an organic supplement restored speed of development and fertility. Again the question remains: do organic substances act directly on the *Artemia* or *via* the food organisms?

New circumstantial evidence makes unlikely the hypothesis of the direct effect of organic nutrients on *Tigriopus* and *Artemia*. Stephens and Schinske (1961) found that most soft-bodied invertebrates can absorb and incorporate from dilute solution radioactive amino acids and sugars. The only invertebrates which had negligible uptake were the Crustacea, probably due to the impervious chitinous exoskeleton. Our experience with the Utah strain of *Artemia* grown in artificial media supports the hypothesis that these animals depend on particulate organic matter as their main source of food, but that they also imbibe very small quantities of liquid.

(a) To obtain *Artemia* juveniles we need ~ 10 mg.% of precipitated albumin and 50 mg.% of insoluble starch. We can replace partly the protein particles with various amino acid mixtures mimicking albumin, liver, etc., but about 300 mg.% of assorted amino acids are needed to replace 5 mg.% of precipitated albumin—hence solutes are taken up but have ~ 1/60 the efficiency of particulates. It was impossible to replace the precipitated albumin completely because the amino-acid mixtures become inhibitory at or above 600 mg.% despite the notorious ability of *Artemia* to grow at ~ 20 g.% total salts.

(b) Most of the vitamins needed by *Artemia* are water-soluble; they are apparently consumed by *Artemia* directly from the solutions and are not appreciably absorbed on the particles of the medium, even during sterilization, because the supernatant of the autoclaved vitamin-containing medium can support growth when added to the particulate of a non-vitamin-containing medium, and not the reverse. The levels of vitamins needed by the Utah strain are in the mg.% range for thiamine, nicotinic, and pantothenic acids and in the 0.1 mg.% range for riboflavin, pyridoxamine, and folic acid. The need for such high concentrations supports the hypothesis that marine Crustacea imbibe small quantities of liquid. Vitamins are generally needed in minute quantities (1–100 μg.%) but apparently are not toxic at several 100-fold concentrations.

Tigriopus can be grown to the second copepodite stage in artificial media. The media are similar to the ones for *Artemia*, differing mainly in the ratios of the various components. The vitamin mixture found adequate for *Tigriopus* in artificial media is extremely concentrated when compared with the vitamin mixture employed to restore fertility of *Tigriopus* grown on *Isochrysis* or *Chroomonas* (Table VII). Hence direct uptake of vitamins by *Tigriopus* is improbable.

These data seem to exclude the possibility that the remarkable effect of organic substances on the two *Artemia* strains at low salinities can be ascribed to the direct absorption by the artemias of 25 mg.% glucose + 1 mg.% urea, and 0.5 mg.% each of yeast extract and Oxoid liver. The effect may be mediated through the algae.

The *Dunaliella* species so far studied require for growth neither vitamins nor organic compounds of P and N: they are photoautotrophic. However, growth of *D. salina* and *D. viridis* is stimulated by yeast extract, brain-heart infusion, and glucose; some amino acids serve efficiently as N sources. *D. viridis* seems more heterotrophic than *D. salina,* though heterotrophy does not sustain growth in darkness of either (Gibor, 1956b). Since organic substances are actively taken up by these two algae, it is possible that they might be concentrated in the algal cells, enriching their value as food, or may have affected the metabolism and chemical composition of the algae. The ability of many algae to concentrate trace metals (Black and Mitchell, 1952), phosphorus (P-luxury consumption) and other nutrients from dilute solutions is well known. Spoehr and Milner (1949) have shown that *Chlorella pyrenoidosa* grown in N-deficient media are low in proteins and rich in fats, the opposite occurs in normal (N-rich) media; changes in cell composition were induced in other algae by nutritional and other cultural conditions (Fogg and Than-Tun, 1960).

TABLE VII

Vitamin mixtures for Tigriopus
(μg. per 100 ml. of final medium)

	Supplement for mono-axenic cultures	For artificial media
Thiamine	61	750
Nicotinamide	6	300
Ca pantothenate	6	375
Riboflavin	0.3	22.5
Pyridoxine	80	150
Folic acid	0.4	52.5
Biotin	4.6	7.5
Putrescine		302
Inositol	9	75
Folinic acid	0.4	

Since the nutritional deficiencies of *Artemia* were evident at salinities below sea water concentration, one wonders whether osmotic stress may have affected directly the algae or the Crustacea or both. Unfortunately our experiments on the nutrition of *Artemia* in artificial media were done at sea water concentration and information is lacking on how salinities affect the nutritional requirements of *Artemia.*

It is known that algal photosynthetic rate and glycerol production are greatly affected by NaCl concentrations: twice as much radioactive C is fixed by *D. tertiolecta* at 14% NaCl than at 0.14% even though the alga had been preconditioned to different salinities by 5 weekly transfers. The quantity of glycerol produced at high salinity is 60 × higher, indicating that the nature of the photosynthate varies with salinity, *i.e.,* more glycerol is produced at high salinity and more polysaccharides at low salinities; but at low salinities C-fixation is twice as low (Craigie and McLachlan, 1964). In our cultures low-salinity stress may have similarly changed the metabolism and cell composition of the preconditioned algae.

Glycerol is an important metabolite in the physiology of the early stages of *Artemia.* Free glycerol synthesis from stored trehalose or glycogen follows rehydration, emergence and hatching of the *Artemia* nauplii. According to Clegg (1964) the amount of glycerol produced by the emerging nauplii increased with increased osmotic pressure of the medium. The glycerol is released into the medium following the hatching of the nauplii. This behavior parallels that of the *Dunaliella* (Craigie *et al.*, 1966). No studies have been done on the synthesis of glycerol by the adult *Artemia* in response to salinity stress.

Finally, osmotic stress may have affected the nutritional requirements of *Dunaliella.* Temperature stress (at lethal temperatures) has been shown to affect widely the nutritional requirements of *Ochromonas* (Hutner *et al.*, 1957), and *Gyrodinium cohnii* (Gold and Baren, 1966), inducing new or higher requirements in vitamins and trace metals. While temperature stress may stop growth of some algae in unenriched media, the growth of *Dunaliella* seemed unaffected or else affected in a minor way after these species were adapted to hyposalinity, *i.e.*, they grew about as well at all salinities. Nonetheless rich organic supplements, *e.g.*, yeast and liver extracts, were needed to restore normal development and fertility in *Artemia.* It seems, then, justified to postulate that hyposalinity may have affected primarily the physiology of the algae, and that the organic supplement somehow restored their nutritional value for *Artemia.*

SUMMARY

1. Two tetraploid strains of *Artemia salina* were axenized. The bisexual strain from the Great Salt Lake was obtained bacteria-free by serial washes of the wintering eggs in Merthiolate. The parthenogenetic strain from the Comacchio salines was axenized by serial washes of gravid females and nauplii in antibiotics; the yeast infection was eliminated by high salinity.

2. Methods for the maintenance of the bacteria-free stocks were defined. Various phytoflagellates were found suitable food organisms for both strains of *Artemia* when grown in appropriately enriched sea water.

3. Two species of *Dunaliella* were adapted to grow in synthetic salt solutions ranging from 0.5 to 20.5% total salts. These conditions permitted the study of the effect of salinity on egg production of *Artemia* in diaxenic culture.

4. Great Salt Lake *Artemia* grown at various salinities, in inorganic enrichments and fed *ad libitum* with salinity-adapted *D. viridis* and *D. salina,* failed to deposit eggs in solutions containing less than 3% salts. The Comacchio *Artemia* was even more sensitive: in low salinities development was inhibited even though the algae were abundant.

5. The reduced fecundity of *Artemia* at low salinities could be reversed by adding organic enrichments, *i.e.*, small quantities of glucose, liver extract, yeast extract and vitamins. The organic enrichment was thought to affect the metabolism of the algae rather than that of *Artemia.*

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EFFECTS OF THE PLEROCERCOID LARVA OF A PSEUDOPHYLLIDEAN CESTODE, LIGULA INTESTINALIS, ON THE PITUITARY GLAND AND GONADS OF ITS HOST

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In Britain the plerocercoid larvae of the pseudophyllidean cestode *Ligula intestinalis* parasitize several species of cyprinid fish (Fig. 1). Since the beginning of the present century it has been recognized that the presence of this parasite in the body cavity of the fish is associated with a suppression of the gonads of the host. This effect is so marked that it is often difficult to distinguish gonadal tissue from surrounding mesenteries, and the sex of the gonad can only be determined by histological examination.

Gonadal suppression in *Ligula*-infected fish has been observed by several authors including Parona and Mazza (1900), Willer (1912), Fuhrmann (1934), Kerr (1948), Voore (1950), Kirschenblat (1951), Jara (1953) and Dence (1958). Kerr extended his observations to include a study of the effects of parasitism on the pituitary gland. In *Ligula*-parasitized roach (*Rutilus rutilus*) Kerr noted that certain basophils of the mesoadenohypophysis were smaller and more sparsely granulated than similar cells in non-parasitized roach. He observed no changes associated with parasitism in other pituitary cells.

An attempt has been made here to extend this work by experimental means.

As described by Arme and Owen (in preparation) *Ligula* has been found in the following fish species in Northern and Central England: roach, rudd (*Scardinius erythrophthalmus*), bream (*Abramis brama*), dace (*Leuciscus leuciscus*) and gudgeon (*Gobio gobio*). Investigations, except where stated, were restricted to the roach.

MATERIALS AND METHODS

1. *Histological procedure*

Fish were measured and weighed, then killed by decapitation. The dorsal and lateral aspects of the brain were exposed and, after removal of the lower jaw, the head was fixed in Bouin's fluid. Decalcification was effected in 5% nitric acid containing 1% urea. Tissues were embedded in either polyester wax (MP 34° C.) or in paraffin wax (MP 56° C.) and serial longitudinal sections were cut at $7\ \mu$. Some pituitaries were dissected out immediately following death and processed as above with the omission of decalcification. No differences in the staining properties of pituitaries prepared by these two methods were observed.

Sections were routinely stained using Mallory's modified triple stain (Mallory, 1936), Heidenhain's azan, the aldehyde fuchsin (AF) technique of Gabe (1953)

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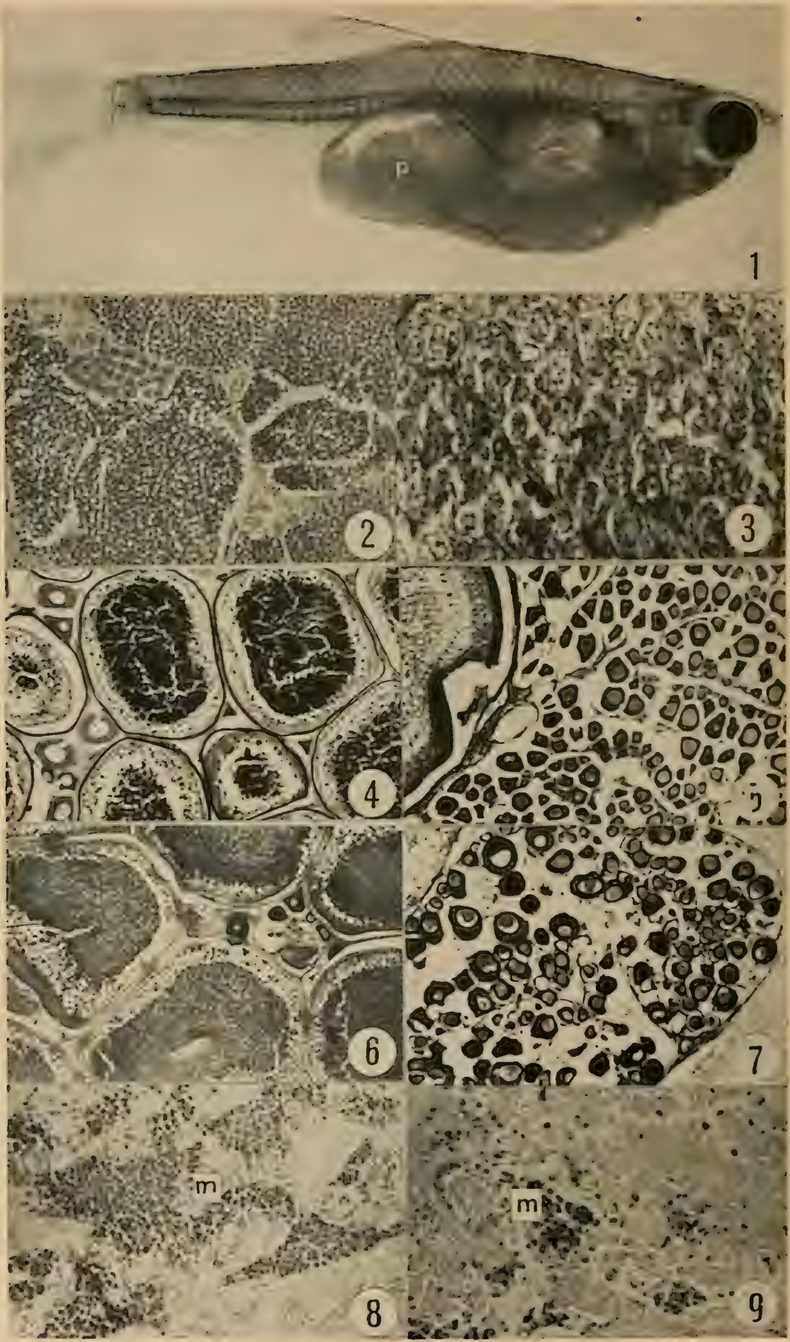
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Note: Figures 2–9 refer to animals examined in the period immediately prior to breeding.

Figures 1–9.

or, following treatment with acidified potassium permanganate, with acidified alcoholic alcian blue (AB) used either alone or in combination with the periodic acid-Schiff (PAS) method. All the basophils of the roach mesoadenohypophysis are AF-, AB- and PAS-positive. However two basophils may be distinguished since one stains more rapidly in alcian blue than the other. Consequently, after 30 seconds immersion in AB, the basophils of one type are sufficiently stained to take up no red color when immersed in PAS, whereas others do so and hence stain purple.

Gonads were weighed and together with thyroid and interrenal tissues were fixed in Bouin's fluid or formol-saline.

Attempts were made to identify the ACTH-secreting cells of the roach pituitary using the lead haematoxylin method of MacConaill (1947). For this purpose both parasitized and non-parasitized fish were killed and fixed immediately following netting in order to minimize possible stress effects due to transportation and handling. No lead haematoxylin-positive cells were found.

The South African clawed toad, *Xenopus laevis*, was used in certain experimental investigations. The histology of the pituitary of this animal was studied by the techniques of Kerr (1965).

2. *Experimental procedures*

Considerable difficulty was experienced in maintaining parasitized roach in laboratory aquaria over extended periods of time. As described by Arme and Owen (in preparation) such fish are affected in various ways in addition to gonadal suppression which may prejudice their chance of survival. Even under natural conditions it is possible that the life expectancy of these fish is short. In many cases the precise cause of death is unknown but many fish suffered incurable infection with the fungus *Saprolegnia parasitica*.

A number of operative procedures proved too lethal for any conclusions to be drawn from the experiments, but these can be listed briefly: gonadectomy; injections of pregnant mare's serum and chorionic gonadotrophin; the removal of plerocercoids from parasitized fish was also unsuccessful because of the extensive proliferation of connective tissue in which the worms become firmly enmeshed.

In the following procedures, involving non-parasitized fish only, survival of experimental animals was generally good.

a. *Implantation of Ligula-plerocercoids*

Animals were anesthetized with MS 222 (Sandoz) and held ventral side uppermost by an assistant. The fish were doused with water from time to time to

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Figure 1. Intact parasitized roach cleared in oil of wintergreen to show plerocercoids of *Ligula intestinalis* in the body cavity—natural size (p = plerocercoids).

Figure 2. Mature testis of non-parasitized roach (× 160).

Figure 3. Testis of parasitized roach containing only spermatogonia (× 230).

Figure 4. Mature ovary of non-parasitized roach (× 28).

Figure 5. Ovary of parasitized roach containing only oogonia and primary oocytes (× 30).

Figures 6 and 7. Ovaries of non-parasitized and parasitized dace (× 30).

Figures 8 and 9. Longitudinal sections of pituitaries of non-parasitized and parasitized roach to show basophils of mesoadenohypophysis (× 90 and 125, respectively). m = mesoadenohypophysis.

remove mucus which tended to accumulate around the mouth and gills. A weighed plerocercoid was introduced into the body cavity *via* a 1- to 2-cm. incision in the ventral body wall. The incision was closed with gut sutures and the fish allowed to recover from the effects of anesthesia in rapidly flowing tap water. Control animals were implanted with either flexible polythene strips, living plerocercoids of *Schistocephalus solidus* obtained from the three-spined stickleback (*Gasterosteus aculeatus*) or freshly heat-killed *Ligula*-plerocercoids.

b. *Administration of steroids*

Pellets of testosterone or oestradiol 17β (Organon) weighing 25 mg. were implanted into the body cavity *via* a 0.5-cm. incision in the ventral body wall; suturing proved unnecessary.

c. *Injection of Ligula-extracts*

Ligula-plerocercoids (11.0 g. fresh weight) were homogenized in 25 ml. saline. The resultant suspension was divided into lots of 0.1 ml. and deep frozen. Non-infected fish were injected twice weekly intraperitoneally; control animals received saline alone.

d. *Implantation of Ligula-plerocercoids into Xenopus laevis*

Anesthesia of male and female *Xenopus* was effected in MS 222; weighed *Ligula*-plerocercoids were introduced into either the dorsal lymph sac or the body cavity. Control animals received polythene strips or living plerocercoids of *Schistocephalus solidus*.

Results

1. *Natural infections*

a. *Gonads*

The size reduction of host gonads is severe in roach, rudd, bream and dace but less marked in gudgeon. As described below it is associated with the inhibition of gamete maturation.

In the non-parasitized roach, immediately prior to the breeding season, the testes are large and in an advanced state of maturation (Fig. 2). In the parasitized male the minute gonads contain only spermatogonia situated in ill-defined lobules bordered by loose sheets of connective tissue (Fig. 3). Maturation stages beyond spermatogonia are totally absent from the testes of parasitized fish at all times of year.

The ovaries of non-parasitized female roach in spring contain many large, yolky oocytes, together with oogonia and oocytes in the first growth phase (Fig. 4). The gonads of parasitized females are small and contain only oogonia and oocytes in the first growth phase (Fig. 5).

A similar marked reduction in gonadal development has been observed in the limited number of rudd, bream and dace which have been examined. Transverse sections of the ovaries of non-parasitized and parasitized dace are shown in Figures 6 and 7. In gudgeon the reduction in gonadal size is less marked than in roach. In parasitized males maturation proceeds beyond the level of spermatogonia but stages further than the secondary spermatocyte level have not been noted. In parasitized female gudgeon fully developed ova have never been observed although in some oocytes there is evidence of the early stages of vitellogenesis.

b. *Pituitary*

Longitudinal sections of the pituitaries of parasitized and non-parasitized roach, rudd and gudgeon are shown in Figures 8–13. The general structure of the gland in all these fish is similar to that described for certain cyprinids by Kerr (1942). In the following description reference is made primarily to the meso-adenohypophysis, since it is the basophils of this region that show marked changes associated with parasitism.

In non-parasitized roach immediately prior to the breeding season (Fig. 8) there are two types of basophil cells of which the more prominent reaches a diameter of 12 to 14 μ and stains various shades of purple following AB-PAS; separate red- and blue-staining granules can be seen in the cytoplasm of some cells. In the majority of these cells the nucleus is surrounded by a distinct nuclear membrane enclosing a clear nucleoplasm and a prominent nucleolus. These basophils are similar in staining properties and distribution to cells thought to be gonadotrophs in other fish species (see Stahl, 1963). The second basophilic cell type is smaller, ranging in diameter from 3 to 7 μ. Its cytoplasm is finely granulated and stains blue, having no affinity for PAS following AB treatment. These cells are histologically identical to basophils found in the proadenohypophysis which in some other fish species are thought to secrete thyroid-stimulating hormone (see Olivereau, 1963).

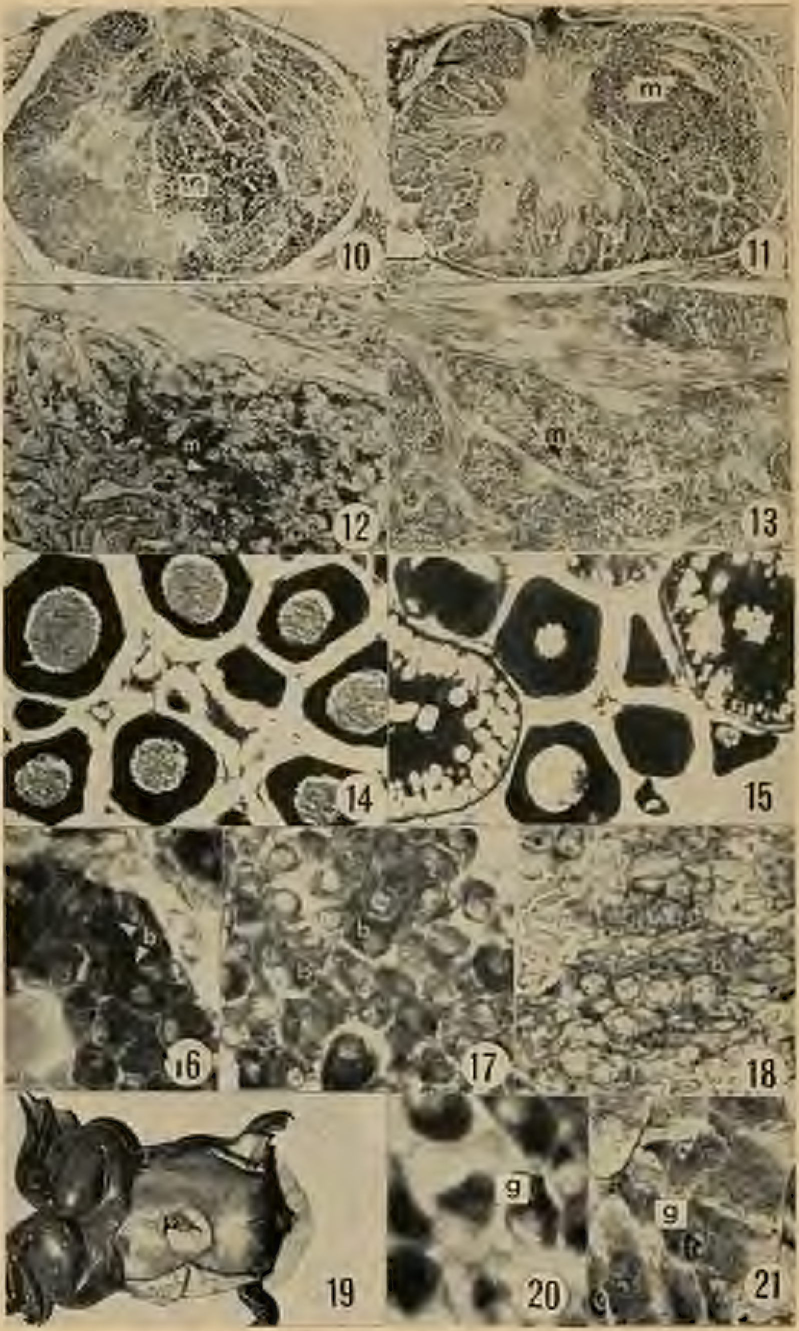
In the spent non-parasitized fish the purple-staining basophils are smaller with a diameter of 5 to 10 μ, and lightly granulated. Following spawning there is a gradual increase in both the size and level of granulation of the purple basophils, culminating in the pre-spawning condition described above. The blue-staining basophils, on the other hand, exhibit no seasonal variation in size or staining properties.

Two basophil types similar in staining properties to those described above are present in the mesoadenohypophysis of parasitized fish although they are much reduced in number (Fig. 9). Further, although there is a considerable range in both the size and level of cytoplasmic granulation in the purple basophils, the majority are lightly granulated and small, rarely exceeding 7 μ in diameter. In many of these cells the nucleoplasm appears opaque, the nuclear membrane is often irregular in outline and a prominent nucleolus is lacking. There is no seasonal variation in the histological appearance of the purple basophils in parasitized fish. The blue staining basophils in parasitized fish appear histologically identical to those found in non-parasitized specimens.

Two similar types of mesoadenohypophyseal basophil have been demonstrated in the pituitaries of rudd, bream and gudgeon. Longitudinal sections of the pituitaries of parasitized and non-parasitized rudd and gudgeon are shown in Figures 10–13. The effects of parasitism appear similar to those described for roach.

c. *Thyroid and interrenal*

A histological examination of these glands in parasitized and non-parasitized roach revealed no differences ascribable to parasitism.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Note: Figures 10–13 refer to animals examined in the period immediately prior to breeding.

Figures 10–21.

2. *Experimental infections*

a. *Implantation of Ligula-plerocercoids into non-infected roach*

All but two of the fish utilized for this procedure proved to be female. Provided that the animals remained free from fungal attack, most survived in an apparently healthy condition for several months after implantation. Parasite growth was noted following implantation in many fish, but in some instances plerocercoids showed a decrease in weight. Worms were usually recovered alive although frequently enmeshed in strands of connective tissue resembling that found in natural infections. Plerocercoids of *Schistocephalus solidus,* used in some control animals, died and were gradually absorbed following encapsulation by the fish.

i. *Gonads*

The ovaries of fish examined six weeks after implantation showed two differing conditions, apparently dependent upon the degree of oocyte maturation at the time of the operation. Twenty fish implanted during the later stages of oocyte maturation and examined up to six weeks later revealed the presence of numerous pre-ovulatory corpora atretica in the ovaries. Each corpus atreticum was surrounded by fusiform cells and the degenerating yolk contained numerous spherical and ovoid bodies similar to those described by Barr (1963) in atretic follicles of the plaice (*Pleuronectes platessa*) and by Arme (1965) in a hermaphrodite specimen of roach. There was a marked increase in stromal tissue in the ovaries of implanted fish. Thirty fish were implanted in the immediate post-spawning period and examined up to three months later. The ovaries of these fish contained only oogonia and oocytes in the first growth phase (Fig. 14). In control fish, although occasional atretic follicles were present and in some instances oocytes tended to be small, gonadal development appeared to be essentially normal and comparable to that which occurred in fish under natural conditions (Fig. 15).

ii. *Pituitary*

Certain histological changes were detected in the pituitaries of *Ligula*-implanted roach. These changes were restricted to the purple-staining basophils of the mesoadenohypophysis and were independent of season. After six weeks the cytoplasm of many of these cells had partially degranulated, the residual granules staining blue and contrasting sharply with the red-purple background staining of the cytoplasm (Fig. 17). Following longer periods of implantation a further

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FIGURES 10 AND 11. Longitudinal sections of pituitaries of non-parasitized and parasitized rudd (× 36).

FIGURES 12 AND 13. Longitudinal sections of pituitaries of non-parasitized and parasitized gudgeon (× 64).

FIGURE 14. Ovary of roach after two months *Ligula* implantation (× 180).

FIGURE 15. Ovary of control roach (× 190).

FIGURE 16. Mesoadenohypophyseal basophils of control roach (× 700).

FIGURE 17. Basophils of roach following six weeks *Ligula* implantation (× 730).

FIGURE 18. Basophils of roach following 3 months implantation (× 730). b = purple staining basophils of the mesoadenohypophysis.

FIGURE 19. *Ligula*-implanted *Xenopus.* Parasite largely intact after seven weeks.

FIGURE 20. Type II basophils of control *Xenopus.*

FIGURE 21. Type II basophils of *Xenopus* following six weeks *Ligula* implantation. g= Type II basophil.

reduction in the level of granulation was observed and the cytoplasm gradually lost most of its affinity for AB. After three months a marked reduction in the cytoplasmic volume had occurred and the cells stained red following AB-PAS treatment, the cytoplasm appearing slightly granular in some cells, homogeneous in others. The cells gave only a weakly positive reaction when stained with AB or AF alone (Fig. 18). In control fish (Fig. 16) the large basophils of the mesoadenohypophysis retained their normal affinity for both AB and PAS.

b. *Implantation of steroids*

In the dosage given, oestradiol 17β proved lethal to roach; all the recipients died within 15 days. In contrast 10 out of 16 testosterone-implanted animals survived for up to three months. All implanted fish were females and changes were detectable in the ovary after six weeks. Oocytes in the vitellogenic phase were atretic while primary oocytes showed a reduction of their affinity for basic stains.

In the pituitary, following testosterone implantation, degranulation and a progressive reduction of affinity for AB was noted in both basophil types in the mesoadenohypophysis. The basophils of the proadenohypophysis were apparently unaffected by testosterone and retained their normal affinity for AB.

c. *Injection of materials*

Parasitized fish were unable to survive repeated injections of the various substances used and death usually occurred within two weeks. However, two parasitized fish, each of which had received a total of six pituitaries from non-parasitized roach, survived for 40 and 47 days, respectively. No changes were noted in the pituitaries of these animals, both females, but histological examination of the gonads showed that vitellogenesis had commenced in some oocytes. Vitellogenesis had never previously been observed in parasitized fish.

Injection of *Ligula*-extracts resulted in no pituitary changes after a period of six weeks although in both experimental and control animals atresia of follicles was observed in females. No gonadal changes were produced in male fish.

d. *Implantation of Ligula plerocercoids into Xenopus*

Ligula-implanted *Xenopus* usually survived for at least three months, after which time experiments were terminated. Plerocercoids placed in the body cavity of nine animals died and had almost disappeared after six weeks. Those placed in the dorsal lymph sac (14 animals) often remained alive for two months (Fig. 19) but after longer periods the worms were recovered dead, often encapsulated in host tissue. Changes attributable to *Ligula*-implantation have been detected in certain cells of the pars distalis designated type II basophils by Kerr (1965) and thought to be gonadotrophs responsible for the secretion of FSH.

As shown in Figure 20 in control animals the type II basophils contain tightly packed granules stainable with AB and AF whereas similar cells in implanted animals showed a marked diminution in the numbers of these granules after six weeks (Fig. 21). After three months little AB- or AF-positive material remained in these cells although their cytoplasm retained a strong affinity for PAS. The appearance of the type II basophils was similar to that described by Kerr (1965) following testosterone implantation.

Gonads of implanted animals were apparently unaffected.

Discussion

Teleost gonadotrophins are thought to originate from certain basophils of the mesoadenohypophysis. Reviews of the relevant literature have been compiled by Pickford and Atz (1957), Dodd (1960), Ball (1960), Stahl (1963) and Barr (1965). Following alcian blue-periodic acid-Schiff (AB-PAS) treatment two basophil types have been demonstrated in the mesoadenohypophysis of roach, rudd, bream and gudgeon. It is suggested that one of these cell types, which stains purple following the above staining procedure, manufactures at least one gonadotrophin. This suggestion is based upon the observation that in roach these basophils undergo cyclical changes in both size and levels of granulation which can be associated with levels of gonadal development. The second basophil type, which stains blue, is histologically identical to basophils of the proadenohypophysis which in some teleosts are thought to be thyrotrophs (see Olivereau, 1963).

The presence of *Ligula intestinalis* plerocercoids in several fish species is associated with a suppression of gamete maturation and with cytological changes in the host pituitary. In naturally infected fish the decrease in the numbers of purple-staining basophils, the marked reduction in both their size and level of granulation and their seemingly inactive nuclei suggest that the presence of *Ligula* is associated with a suppressive effect upon these particular cells. Many investigators have shown that in the absence of pituitary gonadotrophins, full maturation of teleost gametes cannot occur (see reviews cited above). Following hypophysectomy it has been shown that in males the transformation from spermatogonium to spermatocyte is inhibited and in females vitellogenesis in the developing oocyte is arrested. In *Ligula*-infected roach, gamete maturation is arrested at precisely those stages of development where, as described above, the pituitary begins to exert a controlling influence. The effects of parasitism on the gonads of the limited number of rudd, bream and dace which have been examined appear similar to that observed in roach. In gudgeon the effects of parasitism on the gonads are not so marked as in roach but mature gametes have never been observed.

It is possible that *Ligula* produces some substance which, by a depressant effect upon the pituitary function, inhibits development of the gonads, but the nature of this substance is not known. In both the roach and *Xenopus* the results of experimental infections are similar to those which follow testosterone treatment. The parasite may therefore conceivably produce a specific steroid with an anti-gonadotrophic effect upon the pituitary. However preliminary experiments to detect possible steroid activity in the worm, using histochemical methods and the rat vaginal cornification test, have proved negative. It is possible that the parasite may produce a non-steroidal substance with anti-gonadotrophic activity. It has been reported that para-hydroxypropiophenone inhibits gonadotropin secretion in the frog *Rhacophorus schlegelii* (Iwasawa, 1958), and that a similar effect resulted from the treatment of the rat and several other mammals with α-methylallylthiocarbamyl-2-methylthiocarbamoylhydrazine (Paget *et al.*, 1961). Injections of the latter compound into three species of teleost fish were shown by Hoar *et al.* (1967) to block the action of pituitary gonadotrophins on the gametogenetic and endocrine tissues of the testes.

The identification of a substance in *Ligula* with such a specific effect upon the gonadotrophic basophils is clearly of interest and further experiments are being directed towards this end.

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I am especially grateful to Professor J. M. Dodd, Dr. T. Kerr and Dr. R. Wynne Owen for help and guidance.

Summary

1. Infections with the plerocercoid larvae of a pseudophyllidean cestode, *Ligula intestinalis*, are shown to be associated with histological changes in the pituitary gland and gonads of its host.

2. The results of experimental infections in *Rutilus rutilus* and *Xenopus laevis* are similar to those which follow testosterone implantation. It is suggested that the parasite produces an anti-gonadotrophic substance.

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HOLDFAST MOVEMENT IN THE HYDROID CORYMORPHA PALMA: MECHANISM OF ELONGATION

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The hydrozoan stolon appears to provide a rather simple tissue system for studying mechanisms of linear growth. Classically, stolon elongation was thought to result from cell division and tissue growth at the tip (see Kühn, 1914; Berrill, 1961). But recent studies (Overton, 1963; Hale, 1964; Crowell, Wyttenbach and Suddith, 1965; Wyttenbach, 1965) have indicated that cell proliferation and growth are distributed along the length of the stolon, and are absent from the tip. Thus, we must look for a way of explaining how distributed growth is translated into simple elongation rather than into a three-dimensional mass.

To approach this question, I have studied the elongation of a stolon-like tissue, the holdfast of the large solitary hydroid *Corymorpha palma.* These holdfasts are in many ways similar to stolons, and they elongate rapidly into mud on which the polyp lives. Apparently only Torrey (1904) has studied these holdfasts in detail. But with regard to the mechanism of elongation he stated only that it occurs through the activity of amoeboid cells at the tips. In this paper observations are presented which indicate that these holdfasts are elongated by being stretched. They are pulled by their tips, which actively move across a substratum by means of backward-directed waves in the epithelium surface.

Materials and Methods

Polyps of *Corymorpha palma* (Agassiz) were collected on mud flats in Newport Bay, California, where they are locally abundant near and below the low tide mark. The perisarc investing the basal column portion was removed, and the polyps were set on microscope slides in sea water. Holdfasts extended onto the slides in a few hours. For observations under the microscope, the holdfasts were severed adjacent to the polyp (or at other positions) with a sharp scalpel, and a raised coverslip was used to make a horizontal chamber. Time-lapse photography was done using a 16 mm. Bolex camera with a timer produced by Stevens Engineering Company (Newport Beach, California). Cell numbers and distributions were determined in 25 holdfasts on coverslips fixed and stained with hematoxylin. Rates of tissue movement were determined in 6 holdfasts by following, for 2 hours each, successive positions of particular nematocysts and cell junctions, using an ocular micrometer.

In this paper the term "holdfast" refers to the stolon-like outgrowths from the *Corymorpha* polyp. Other terms, including "frustules," "stolons," and "rhizoids," have been used by other workers (see Torrey, 1904) to designate these same structures.

RESULTS

Structure of Corymorpha palma and holdfasts

The stalk of the solitary *Corymorpha palma* polyp (Fig. 1a) is filled by large vacuolated cells. The hydrocoel consists of 10–30 longitudinal peripheral channels which appear as white stripes along the sides of the polyp (Fig. 1). Initiating holdfasts appear as villus-like protrusions in a zone near the basal end of the stalk (Fig. 1b). They are arranged in longitudinal rows, one along each side of every gastrodermal canal.

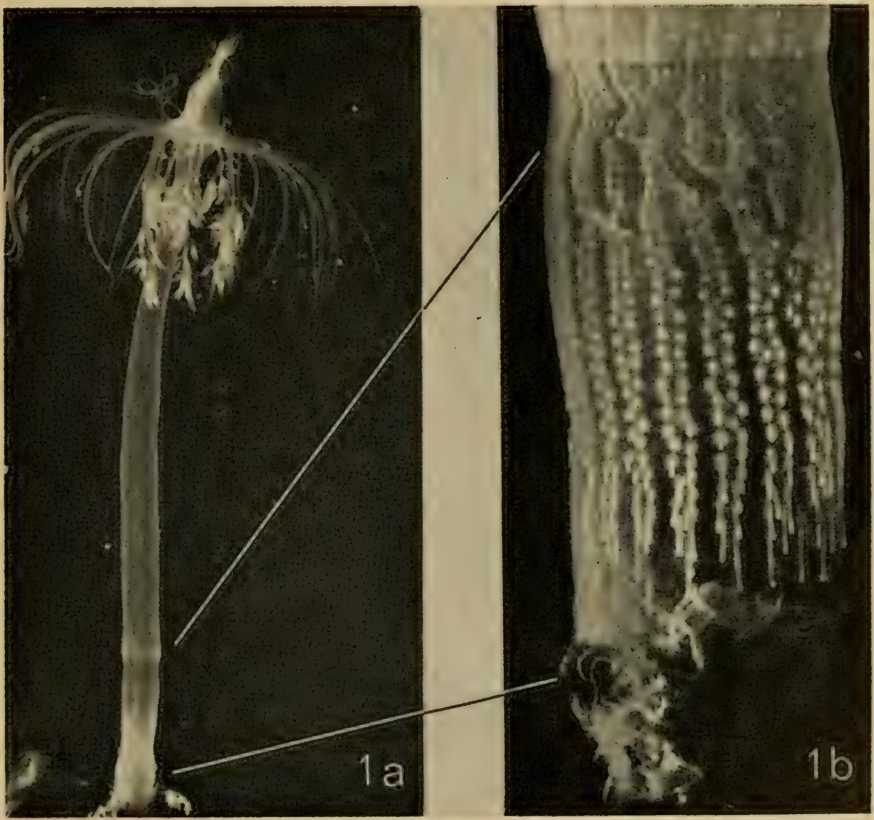
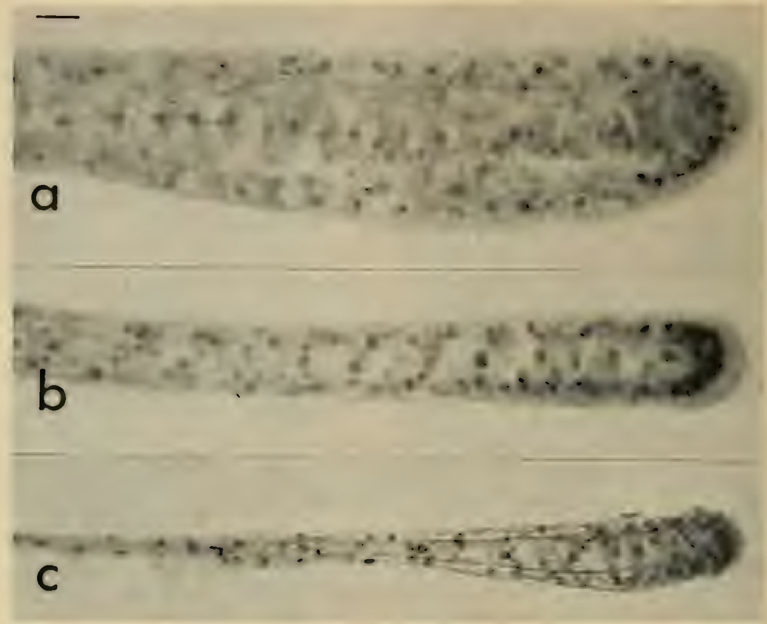


FIGURE 1a. *Corymorpha palma.* Natural size.

FIGURE 1b. Basal region of another polyp, showing gradient in holdfast development. White spots in center of photograph are young holdfast initials. White elongations in lower half of photograph are mature holdfasts beginning to elongate downward along polyp stalk. × 18.

Within this zone of formation, a gradient exists in holdfast development (Fig. 1b), from the top of the region where holdfasts are just being initiated, to the lower part where they have begun to elongate along the polyp stalk and into the mud. Holdfasts originating below this have lost contact with the polyp. Thus, the region of holdfast initiation is continually moving upwards along the body tissues, as mature structures break away from the stalk base, and as new ones begin to form at higher levels. Presumably this movement is compensated by stalk elongation and tissue loss at the polyp base, as is the progression of the budding zone in hydra (Brien and Reniers-Decoen, 1949; Campbell, 1967); the relative position of holdfast initiation remains stationary along the polyp axis.

The cytological events of holdfast initiation have been described by Torrey



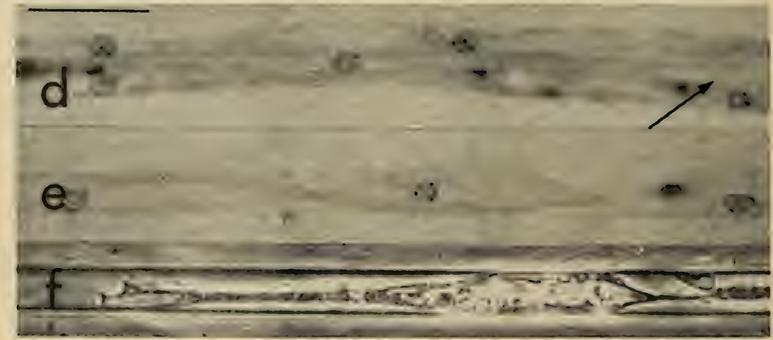


Figure 2 a–c. Tips of young, older, and very old holdfasts, respectively, at same magnification. Line above letter "a" represents 20 μ.

Figure 2 d–f. Trailing portion of a holdfast similar to the one shown in 2b. 2d; just behind tip, where endoderm is still visible (arrow). 2e; further from tip, where cells are arranged in a single column; 2f (phase contrast) near polyp base, where cells are spindle shaped. At left, the cell tip has lost contact with the adjacent cell (not shown). Magnification of 2 d–f; line above letter "d" indicates 20 μ.

(1907). First a plaque of glandular, acidophilic ectodermal cells becomes evident. Endodermal cells subsequently sink into this plaque, forming an ectodermal villus with an endodermal core. Such a villus grows to a length of 3–6 mm. and consists of about 30 endodermal and several hundred ectodermal cells. It does not change shape further unless it comes into contact with a substratum, at which time the tip advances and the villus elongates rapidly. Holdfast structure changes as the tip advances. The tip remains similar to the villus-like initiating structure. But behind the tip, the distinction between endoderm and ectoderm becomes obscured (Fig. 2d, e), as the cells form a narrower column two or three cells thick. Further from the tip, spindle-shaped cells are arranged in a single row. They appear to be stretched, and discontinuities in cell chains frequently occur (Fig. 2f). The perisarc enables the holdfasts to continue functioning as anchoring structures.

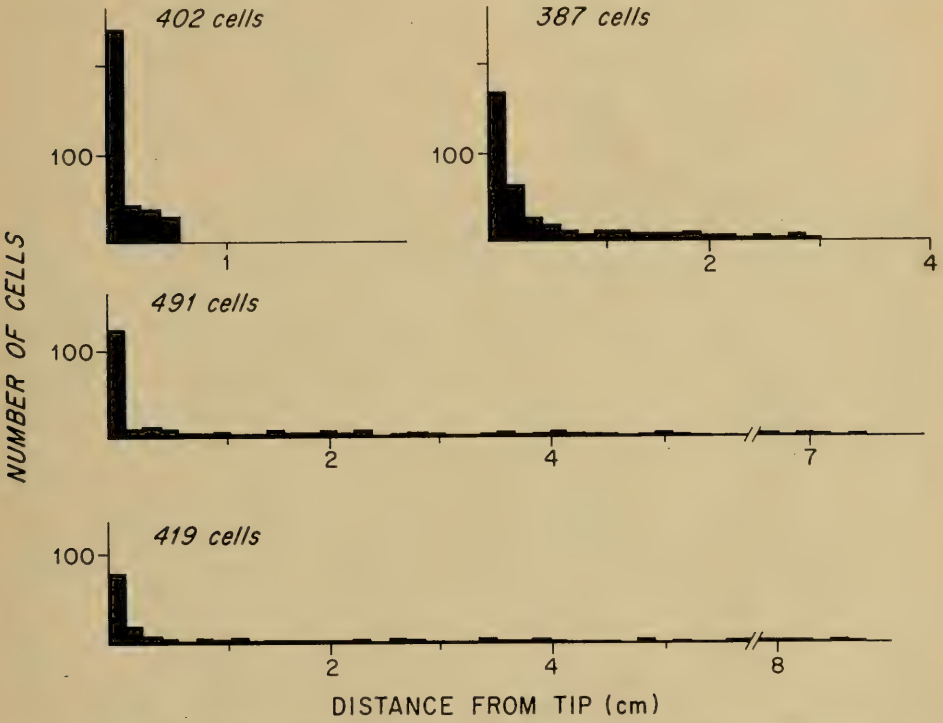


FIGURE 3. Cell distribution along four holdfasts of different lengths (from a single polyp). The total number of cells in each holdfast is shown above graphs.

As the holdfast becomes longer, the size and number of cells in the tip decrease (Figs. 2a–c; 3). In a single polyp, the total number of cells in holdfasts of varying lengths is about the same (see Fig. 3) although there are marked differences in holdfast size from one polyp to another. No mitotic figures have been seen in histological preparations. Apparently, the elongation of a holdfast is not dependent upon cell proliferation.

Holdfast elongation

As a holdfast elongates, tissue all along its length advances. The rate of movement in different portions is shown in Figure 4 (solid lines). The tip moves most rapidly, and the polyp end most slowly: if the holdfast has not broken, tissue in the end of the holdfast joining the polyp does not move, indicating material is not being withdrawn from the stalk. From the rates of movement measured at different holdfast positions it is possible to estimate the rates of tissue elongation along the holdfast (Fig. 4, dotted lines). Tissue within the tip does not elongate, but most or all of that in the trailing portion does. Elongation is most rapid shortly behind the tip where the holdfast is still rather thick (Fig. 2d). Tissue elongation is relatively slow further from the tip where cell junctions have already been very strained (Fig. 2e, f).

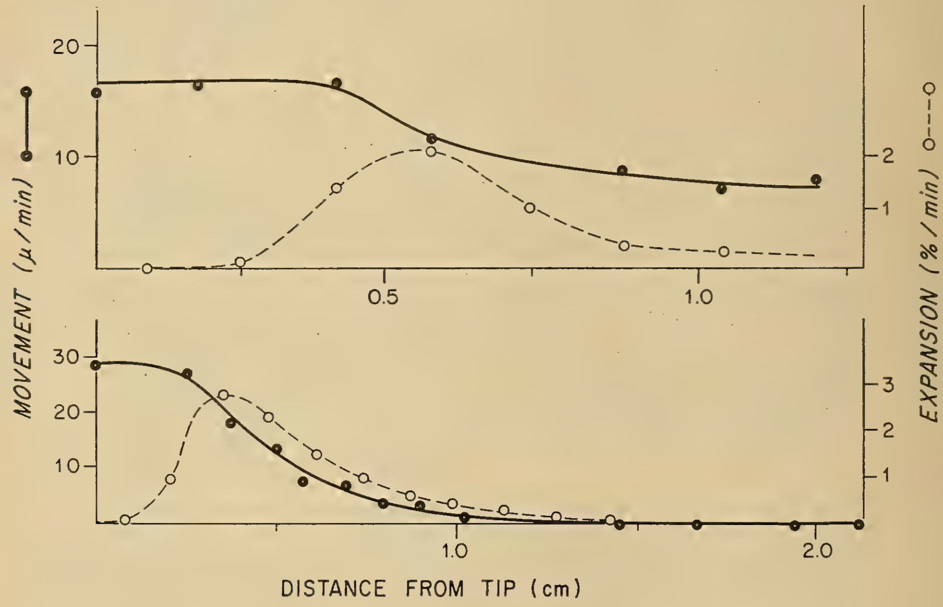
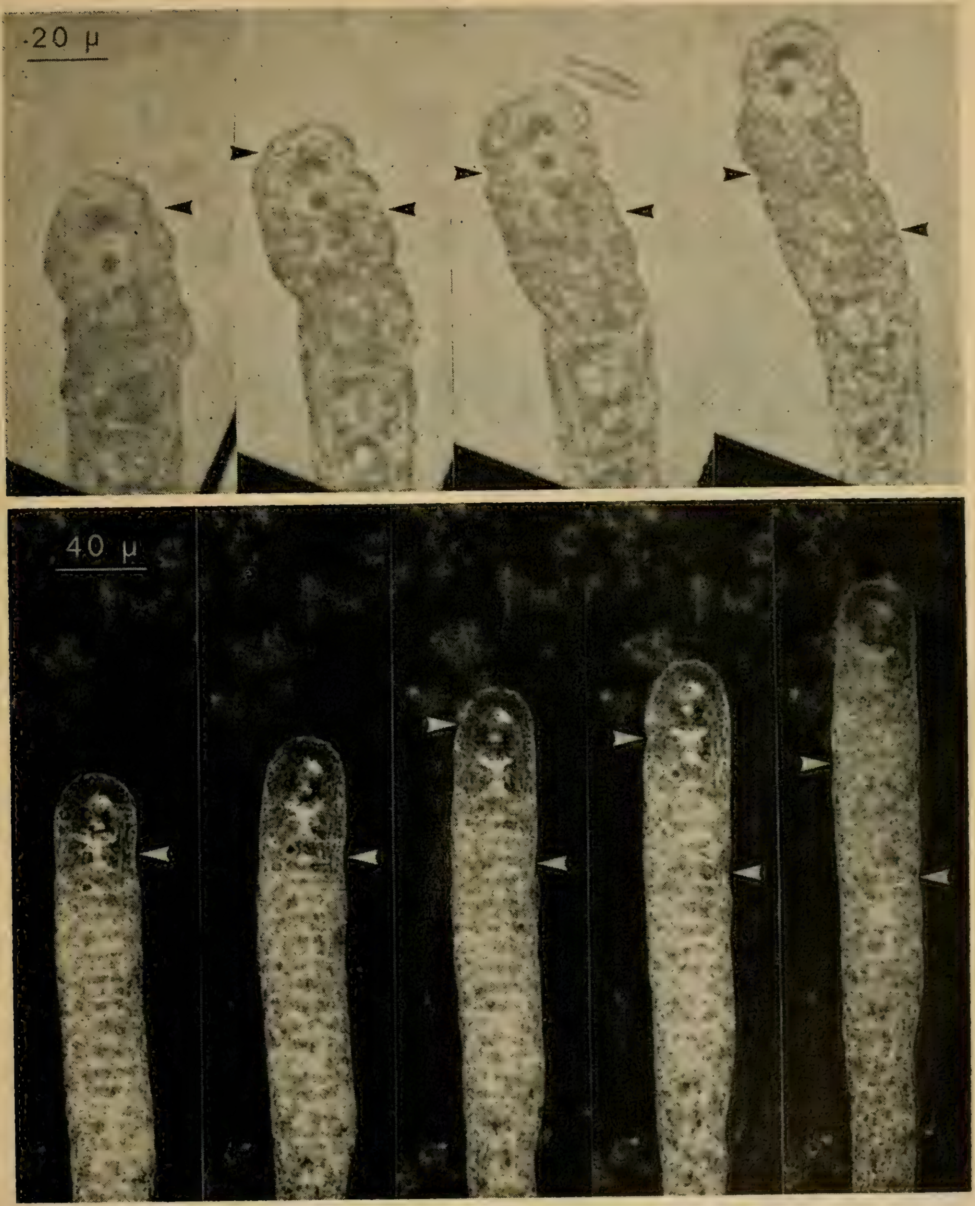


FIGURE 4. Measured rates of cell movement (solid lines) and estimated rates of tissue expansion (broken lines). Holdfast represented at top had broken its connection with the polyp; holdfast represented below was still attached to polyp. Tissue expansion curves represent the axial derivative of the movement curves.

Observations were made on holdfast elongation with time-lapse cinematography. They revealed two basic patterns of tissue activity. One is related to the continuous forward progression of the tip. Waves of surface bulges and furrows move backwards along the tissue at approximately the rate at which the tip advances. Old holdfast tips have annular waves encircling the holdfasts (Fig. 5). In tips of younger holdfasts, the waves rarely completely encircle the stolon, but appear rather as local irregularities in the tip profile (Fig. 6). They may occur as extremely small "ripples" which are very difficult to see except with darkfield



FIGURES 5 AND 6. Time-lapse photographs of advancing holdfast tips, printed from 16 mm. film. Arrows indicate surface irregularities which progress across tissue from the tip. Figure 5; Very old tip. Each interval, 2.4 minutes. Figure 6; Young tip. Each interval, 5 minutes.

illumination. These different forms of waves are probably responsible for the advancement of the tip. They do not merely represent tissue distortions caused by irregularities in the substratum, since the waves move very slowly backwards with respect to the substratum (Figs. 5 and 6).

The second tissue activity of the holdfast tip is a "pumping" motion in which the endoderm alternately moves forward and backward. The periodicity of this movement, which is not observable in all holdfasts, is usually 1–3 minutes. In tips of young holdfasts, where surface waves are comparatively small, there is no apparent relation between movement of the holdfast tip and endodermal "pumping"; the ectoderm moves forward at a more nearly constant rate while the endoderm oscillates. In old holdfast tips, however, the formation of surface waves is related to endodermal "pumping." When the endoderm moves forward a terminal bulge appears, which then progresses backwards from the tip as successive bulges are formed.

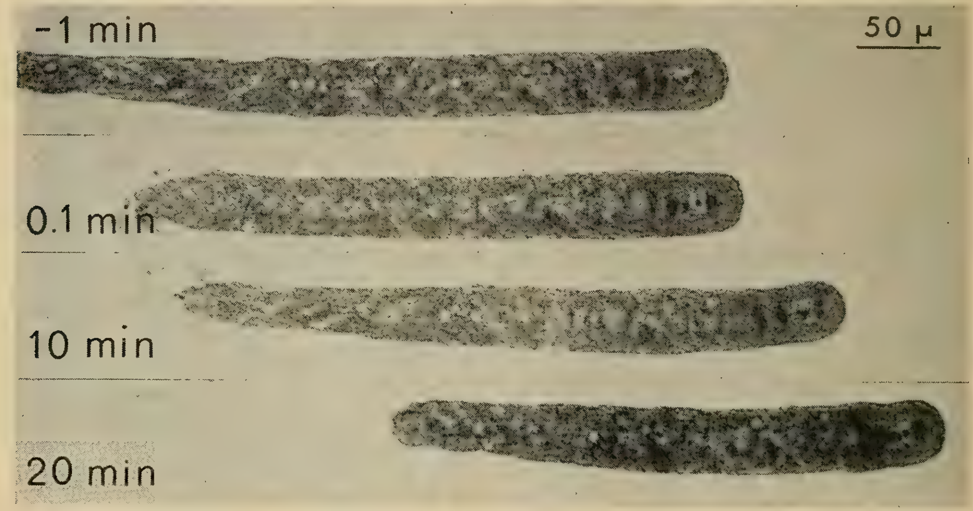


FIGURE 7. Holdfast tip before and after it was cut from trailing tissue. Time is measured from the time of isolation. The rates of tip advance before and after isolation were 6.1 and 6.6 μ/min., respectively.

The surface activity discussed above is restricted to the holdfast tip; the waves cease to be visible behind the region where endoderm and ectoderm are no longer clearly distinguishable.

The ability of the holdfast tip to actively move across a substratum is most strikingly illustrated by the activity of isolated tips. When severed from the trailing holdfasts, tips continue to progress in the same manner as connected tips do (Fig. 7). Terminal pieces as short as 50 μ (about one-quarter the length of a young tip) will sometimes continue to migrate. Tip movement, then, is not dependent upon the trailing holdfast. Frequently a tip moves more rapidly after isolation from the holdfast (as is the case in Fig. 7).

DISCUSSION

The observations discussed in this paper indicate that the elongation of a holdfast is due to the active movement of the tip across a substratum, which stretches the rest of the tissue into a long trail. The stretching and even breaking of cell contacts along the holdfast (Fig. 2) otherwise is difficult to explain. That the tip can move itself is shown by the behavior of isolated holdfast tips. Apparently as the tip migrates forward, a trail is pulled from its most proximal portion, so the tip decreases in size. In broad terms this agrees with Torrey's (1904) conclusion that the tips are pulled forward by the activity of amoeboid cells. But the motivation apparently is derived from the backwards propagation of surface irregularities by the coherent ectoderm.

Do other hydrozoan stolons elongate by mechanisms similar to that in *Corymorpha?* *Corymorpha* holdfasts are unlike "typical" hydrozoan stolons in some respects. They are not hollow, and they have statocysts at their tips (unpublished observations). On the other hand, they resemble other stolons in their ectodermal and endodermal origin from stalk tissue, in their position at the base of the polyp, and in their linear organization. Thus it is at least worthwhile to investigate the possibility that their mechanisms of elongation resemble those of typical stolons, even if their homology with other stolons is questionable.

There are indications that stolons of other hydroid species elongate at least partly by means of terminal behavior rather than by means of terminal growth. Cell division has been found distributed along stolons but absent at their tips in *Cordylophora* (Overton, 1963); *Proboscidactyla* (Campbell, unpublished) and *Clytia* (Hale, 1964). Marking experiments also demonstrate that stolon elongation occurs distant from the tip and that the tip itself neither lengthens nor adds new tissue at its tip (Overton, 1963; Hale, 1964; Crowell, Wyttenbach and Suddith, 1965; Wyttenbach, 1965). The shapes of stolons frequently indicate that they are expanded by stretching rather than through internal growth; for example, some stolons are narrower than the perisarc tubes which invest them, except at the tips. Finally, the stolon tip of at least one other hydrozoan, *Proboscidactyla flavicirrata,* has been found capable of movement across a substratum after it is severed from the rest of the stolon, similar to the behavior of severed *Corymorpha* holdfast tips described in this paper (Campbell, 1966, and unpublished observations).

Many stolons do not elongate along a solid substratum. Some course through water away from a substratum (*Corymorpha* holdfasts can do this slowly and irregularly) and *Hydractinia* stolons will elongate on an air-water interface (unpublished observations). It might be thought that this demonstrates that stolon elongation cannot occur by means of stretching by the tip, since the tip must transfer momentum to some material other than itself. Water seems an unlikely candidate because of its low viscosity. However, most stolons are invested in a perisarc, generally nearly to the tip, and this may provide an effective substratum for the tip to work on.

The elongation mechanisms of fluid-filled stolons are probably not identical to those observed in *Corymorpha* holdfasts. Time-lapse cinematography (Hauschika, 1944; Fulton, 1963; Campbell, unpublished) and direct observation (Berrill, 1949; Hale, 1964) reveal peristaltic contractions of the coenosarc as well as contractile activities of the stolon tip. These are quite complex (Fulton, 1963), but the peristaltic waves appear to be generated in a region slightly behind the stolon tip (Berrill, 1949; Fulton, 1963; Hale, 1964) instead of at the tip as in *Corymorpha* holdfasts. The distribution of stolon expansion is similar in the stolon and holdfasts, but somewhat more complex in the bi-layered stolons (Hale, 1964). In addition, rhythmic fluid movements in stolons may be responsible for the rhythmic distension, and hence extension, of the stolon tip, although isolated tips continue much of their contractile behavior (Berrill, 1949; Hale, 1964; Wyttenbach *et al.*, 1965). Even if hydrostatic distension is a cause of stolon extension, the activities of the tip may still be playing a crucial role in translating pulsations into forward progressions.

Thus, the ordered morphogenesis of holdfasts, and perhaps of stolons, may be directly due to cell and tissue behavior rather than to cell proliferation and growth.

SUMMARY

1. Stolon-like holdfasts in the hydroid *Corymorpha palma* elongate along a substratum. This occurs rapidly and without an increase in cell number.

2. Holdfast elongation represents tissue stretching by the holdfast tip, which actively moves across the substratum. Holdfast tips show the migratory capacity even after they are severed from the trailing tissue. Their movement apparently is due to proximally directed waves in the tissue surface which push against the substratum.

3. Holdfast elongation thus represents an example of patterned morphogenesis which is not dependent upon cell proliferation. It is possible that typical hydrozoan stolons similarly elongate at least partly by means of terminal behavior, rather than by cell proliferation at the tip.

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FREE AMINO-ACID LEVELS IN THE COELOMIC FLUID AND BODY WALL OF POLYCHAETES[1]

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In a previous study of the coelomic fluid of the polychaete, *Nephtys hombergi,* unusually high levels of free amino acids and other ninhydrin reactive nitrogenous compounds were found (Clark, 1964). Insect hemolymph (Wyatt *et al.,* 1956; Duchâteau and Florkin, 1958; Stevens, 1961), sipunculid coelomic fluid (Virkar, 1965) and, to a lesser degree, *Mytilus* blood (Lange, 1963; Bricteux-Grégoire *et al.,* 1964a) are the only other invertebrate body fluids in which similarly large quantities of free amino acids have been reported. It therefore seemed of interest to determine whether the high amino-acidemia observed in *Nephtys hombergi* is a general phenomenon among polychaetes. The opportunity to spend a summer at the Friday Harbor Laboratories provided a chance to examine several species of worms.

It is also known that low molecular weight organic nitrogen compounds contribute a large proportion of the total intracellular osmotic activity in many invertebrates, including polychaetes (see Florkin *et al.,* 1964, for literature review). For this reason, the levels of free amino acids in the body wall of the worms were also investigated. In a subsequent paper are reported the results of a survey study of the effects of osmotic dilution on the levels of free amino acids in both body fluid and tissues (Clark, 1968).

MATERIALS AND METHODS

Animals

Except for a few observations made on *Nephtys hombergi* collected in the Bristol Channel, all the data were obtained on polychaetes collected either intertidally or subtidally in and around San Juan Island, Puget Sound, between June and August, 1965. The choice of species was obviously limited by availability and size of the animals, but an attempt was made to sample as many different types of animals as possible with respect to both morphology and environment. Altogether, fourteen species in eight families have been studied, ten in detail. These include Nephtyidae: *Nephtys caeca* (dredged), *N. caecoides* (False Bay), *N. ciliata* (Minnesota Reef); *N. hombergi* (Bristol Channel); Nereidae: *Nereis vexillosa* (Minnesota Reef, False Bay); Eunicidae: *Diopatra ornata* (False Bay), *Eunice biannulata* (San Juan Point); Orbiniidae (= Ariciidae): *Naineris laevigata* (Kilpatrick's Beach);

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Arenicolidae: *Abarenicola pacifica* (Argyle Bay); Terebellidae: *Amphitrite cirrata, Neoamphitrite robusta, Thelepus crispus* (all Minnesota Reef); Aphroditidae: *Aphrodite japonica* (dredged); Opheliidae: *Travisia pupa* (dredged).

The animals were kept in the laboratory without food, in tables of running sea water (30 to 32 parts per thousand salinity) at 8 to 10° C. They were generally used within a week of being collected. Animals normally occurring in tubes were generally removed from these (exceptions are noted in the data).

Removal of body fluid

The worms were generally killed by very brief immersion in 95% ethanol, although this was not necessary for the less active terebellids, or for *Aphrodite* or *Travisia.* After rinsing in sea water, the worms were blotted dry on filter paper. Coelomic fluid was then removed by successive puncture of the body wall with fine glass pipettes (see Clark, 1964) or, in the case of the terebellids, by direct incision of the body wall. Punctures were made through the thinnest region of the body wall, to minimize damage to the muscle tissue. Extreme care was always taken to avoid contamination of samples with gut fluid. In some species admixture of blood was unavoidable. The likely consequences of this are discussed later. The pooled coelomic fluid from each group of animals was collected in chilled tubes, centrifuged, and an aliquot of the cell-free supernatant taken for preparation of an 80% alcoholic protein-free filtrate. Five volumes of 96% ethanol were added, and after 15 minutes to allow flocculation, these were centrifuged and the supernatant stored at 4° C. until analyses were made.

Coelomic fluid was removed from different regions of some animals to determine whether internal gradients exist. In particular, coelomic fluid from the proboscis region was compared with that in the remainder of the body in *Nephtys* and *Nereis,* and the trunk and tail regions of *Abarenicola* were separately sampled.

When sample volume permitted, estimates of pH of coelomic fluids were made using a Beckman Zeromatic pH meter.

Body-wall samples

After the coelomic fluid was collected from all the animals in each group, a piece of body wall was rapidly removed from each worm. These were taken from the same area in all worms of any one species and contained only body-wall muscle and epidermis. Where possible, the cuticle was removed (*Travisia, Aphrodite*). Each piece of tissue was rinsed very briefly in sea water to remove coelomic fluid and thoroughly blotted on filter paper. It was divided into two halves, one placed on a pre-weighed dish, the other in a pre-weighed test tube for sonification. The tissues were placed in a humid container at 4° C. while body-wall samples from other groups of worms being studied simultaneously were obtained. To check whether this resulted in any significant alteration in the level of free amino acids in the samples, three sets of tissue samples taken from each of three different groups of *Nereis* were treated in the following ways: one sample was immediately sonified (see below), the second was stored one hour at 4° C., then sonified, and the third was kept cold for 4 hours before sonification. In all cases, the amino-acid levels found agreed within the experimental error and there were no consistent trends either toward increase or decrease of amino-acid levels. Since with one or two exceptions, noted in the data, experimental tissues were never stored for more than 1 hour prior to sonification, it would seem unlikely that significant changes occurred in the tissues during this stage.

All the wet tissues were weighed on a Mettler semi-micro balance Type H 16. For dry weight determinations, one set of tissue samples from each group was dried in a desiccator to constant weight. To the tissue samples in pre-weighed test tubes were added 5 ml. distilled water. These were chilled in an ice bath and sonified. Occasionally parts of the cuticle and of the fibrous sub-epidermal basement membranes did not sonify, but microscopic examination in these instances revealed no intact cells remaining. Because of the presence of non-cellular material, all data on body-wall samples are expressed on the basis of tissue water rather than tissue wet weight. Immediately after sonification 2-ml. aliquots of the breis were diluted with 5 parts of 96% ethanol and, after precipitation of the high-molecular-weight components, the samples were centrifuged and the supernatants stored at 4° C. Since the time required for weighing, sonifying and precipitating samples from several groups of animals could require up to 30 minutes, another test was run to determine any effects of tissue storage at room temperature at this stage. After sonification of three samples of *Nereis* body-wall tissue, three aliquots of each were taken for alcoholic precipitation, one immediately, the second after one hour and the third after 4 hours. Again the amino-acid levels in all three aliquots of each sample agreed within experimental error and no trends were observed.

Analytic methods

The ninhydrin micro-method used for the estimation of total ninhydrin-positive nitrogen (Nin + N) has previously been described (Clark, 1964). Double strength citrate buffer was used throughout. Measurements were repeated until replicates agreed to ±0.010 O.D. unit. Proline does not react in this method and no separate analysis was made although it is frequently an important amino acid in invertebrates (Awapara, 1962; Clark, 1964). Therefore, Nin + N levels reported here are low by the proline value.

Aside from the amino-acid derivative taurine, the only non-α-amino compound which reacts quantitatively with ninhydrin under these conditions is ammonia (see Williams *et al.*, 1965). Separate determinations for ammonia nitrogen (NH_3 N) were therefore made on each sample using the micro-diffusion technique of Conway and O'Malley (1942). Titrations were made from a microburette (capacity 1 ml.) using 7.14×10^{-4} N H_2SO_4 (1 ml = 10 μg. NH_3 N), employing a mixed indicator. Replicates were continued until agreement within 0.1 μg. was obtained, at the limit of sensitivity of the method.

Amino-acid nitrogen (AAN), including taurine, was obtained by difference.

RESULTS

Coelomic fluid pH

The pH of polychaete coelomic fluids generally falls between 6.3 and 7.5, that of the sub-tidal species generally being on the alkaline side of neutrality (Table I). In all three species of *Nephtys* and in *Nereis vexillosa* there is an internal pH

TABLE I

Coelomic fluid pH of various polychaetes. Where several regions of the body have been sampled from the same group of worms, this is indicated by ditto marks in the column headed "Number of worms."

Species	No. worms	Region	pH
Nephtys hombergi	48	anterior 1/3	6.51
	"	middle 1/3	6.42
	"	posterior 1/3	6.32
Nephtys caeca	2	proboscis	7.60
	"	anterior 1/2	7.60
	"	posterior 1/2	7.45
Nephtys ciliata	19	proboscis	7.30
	"	all body	7.03
	6	proboscis	7.55
	"	anterior 1/2	6.80
	"	posterior 1/2	6.58
Nereis vexillosa	8	proboscis	7.50
	"	all body	6.97
Diopatra ornata	8	all body	7.14
	25	all body	6.88
Nainereis laevigata	60	all body	6.95
Amphitrite cirrata	1	thorax	6.50
	1	thorax	6.72
Neoamphitrite robusta	1	thorax	7.64
Thelepus crispus	1	thorax	6.35
	5	thorax	6.35
Abarenicola pacifica	7	trunk	7.30
	"	tail	7.49
Aphrodite japonica	1	all body	7.49
	1	all body	7.42
Travisia pupa	1	all body	7.23

gradient of about 0.2 pH units, the anterior end having a higher pH than the posterior (Fig. 1). In the case of *Abarenicola pacifica*, the trunk is more acid than the tail, but since only one measurement is available from coelomic fluid pooled from seven animals this may not prove to be a consistent gradient.

Coelomic fluid and body-wall NH_3 N and AAN

Estimates of ammonia and amino-acid nitrogen were made on the coelomic fluid of 13 species and on the body wall of 10 species of worms. The results are given in Table II and Figure 2. In the table, the data are expressed as mg. N/100

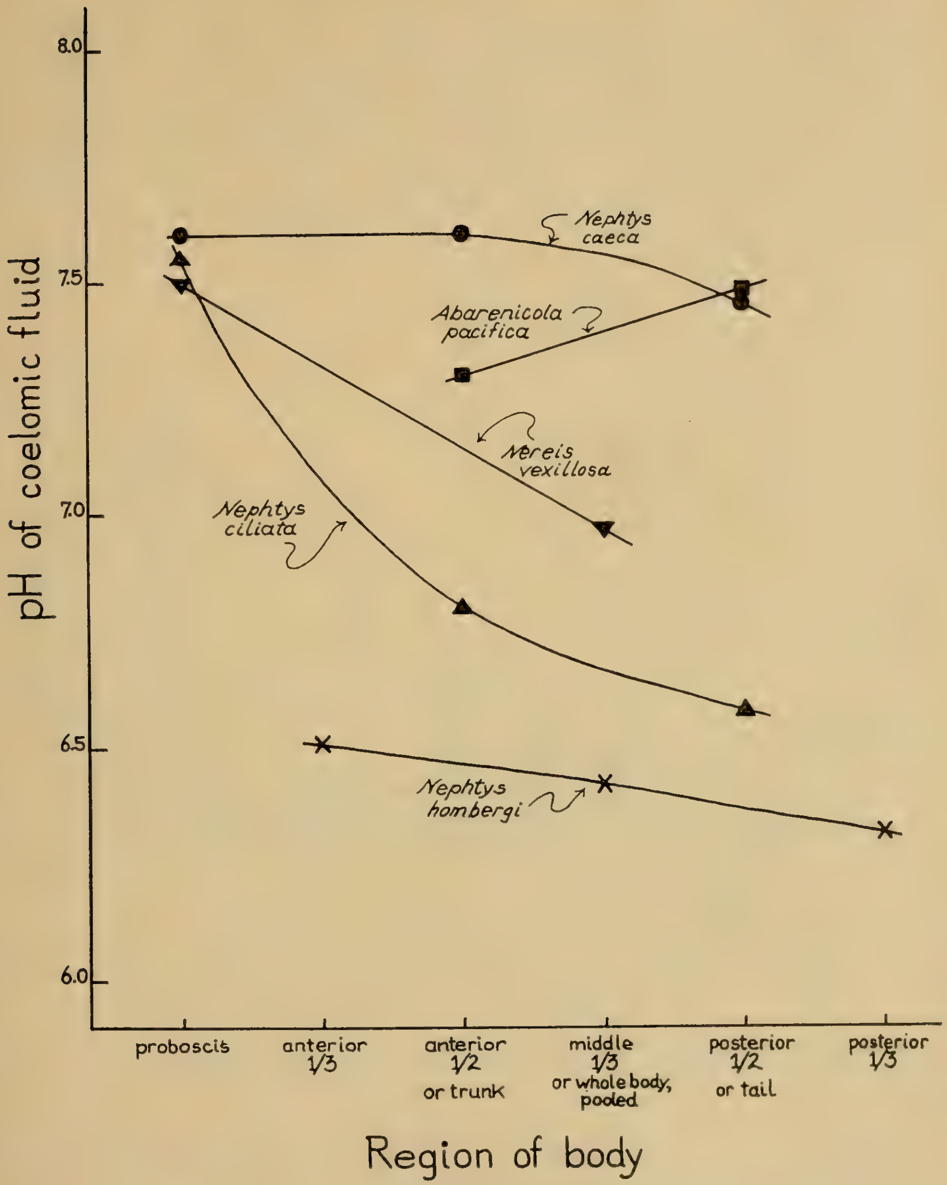

FIGURE 1. The pH of coelomic fluid from different body regions.

ml. coelomic fluid or mg. N/100 g. tissue water, respectively, since these are most frequently met in the literature. In the figure, data are given in m*M* AAN/l. coelomic fluid or m*M* AAN/1000 g. tissue water, which is more meaningful in terms of osmotic activity.

The body-wall AAN values, in all cases, are considerably higher than those in the coelomic fluid. In *Thelepus crispus,* with the highest coelomic fluid AAN values, the difference is by a factor of 4; in *Aphrodite japonica,* with the lowest, it is by a factor of 100. The body-wall values thus show a much smaller relative difference between species than do coelomic fluid values. There is no apparent correlation between the AAN-levels in the body wall and either the phylogenetic relations or environmental conditions of the various species. Differences are as great among the terebellids, or between the closely related nephtyids and nereids as between other groups. The low levels found in subtidal *Aphrodite* are not shared by the equally subtidal *Travisia.*

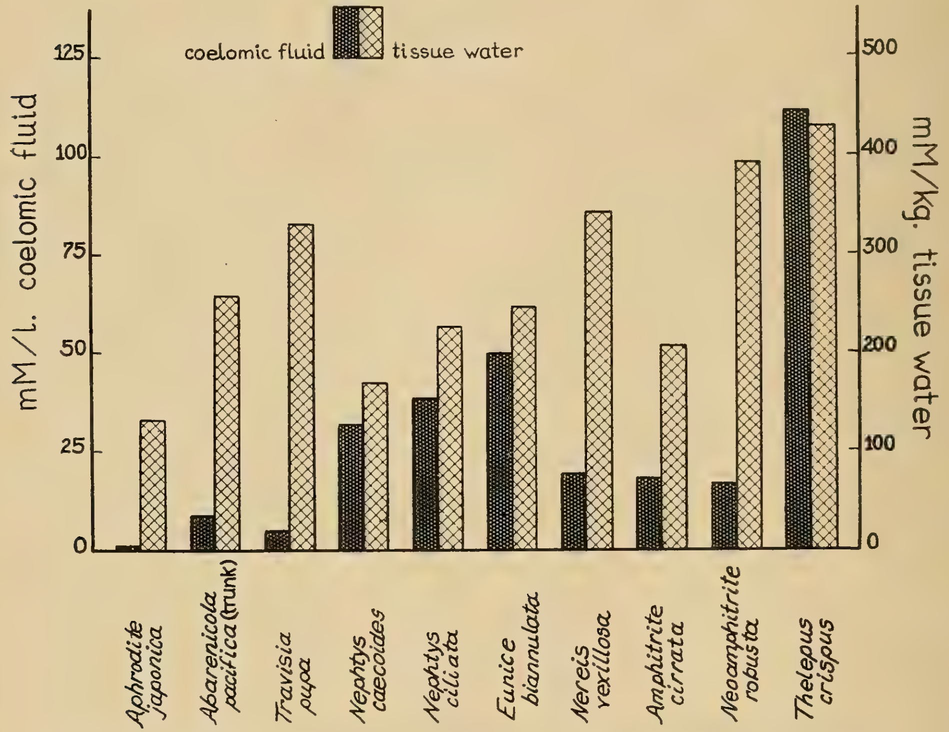

FIGURE 2. Amino-acid nitrogen (AAN) levels in coelomic fluid and body wall of polychaetes, averaged from Table II.

The values of NH_3 N in the body wall are rather variable, but never exceed 10% of the AAN level. In the coelomic fluid, NH_3 N is 5% or less of the AAN level. Whether in fact the body-wall NH_3 N levels are as high as reported is uncertain. The higher levels may only indicate a higher rate of post-mortem intracellular ammonia formation. In two instances, when body-wall samples were left at room temperature for several hours, the NH_3 N values were markedly increased, showing that breakdown does occur in the tissues (Table II).

The coelomic fluid AAN levels are more variable both within and between species. The intraspecific variation may in part depend on the proportion of the

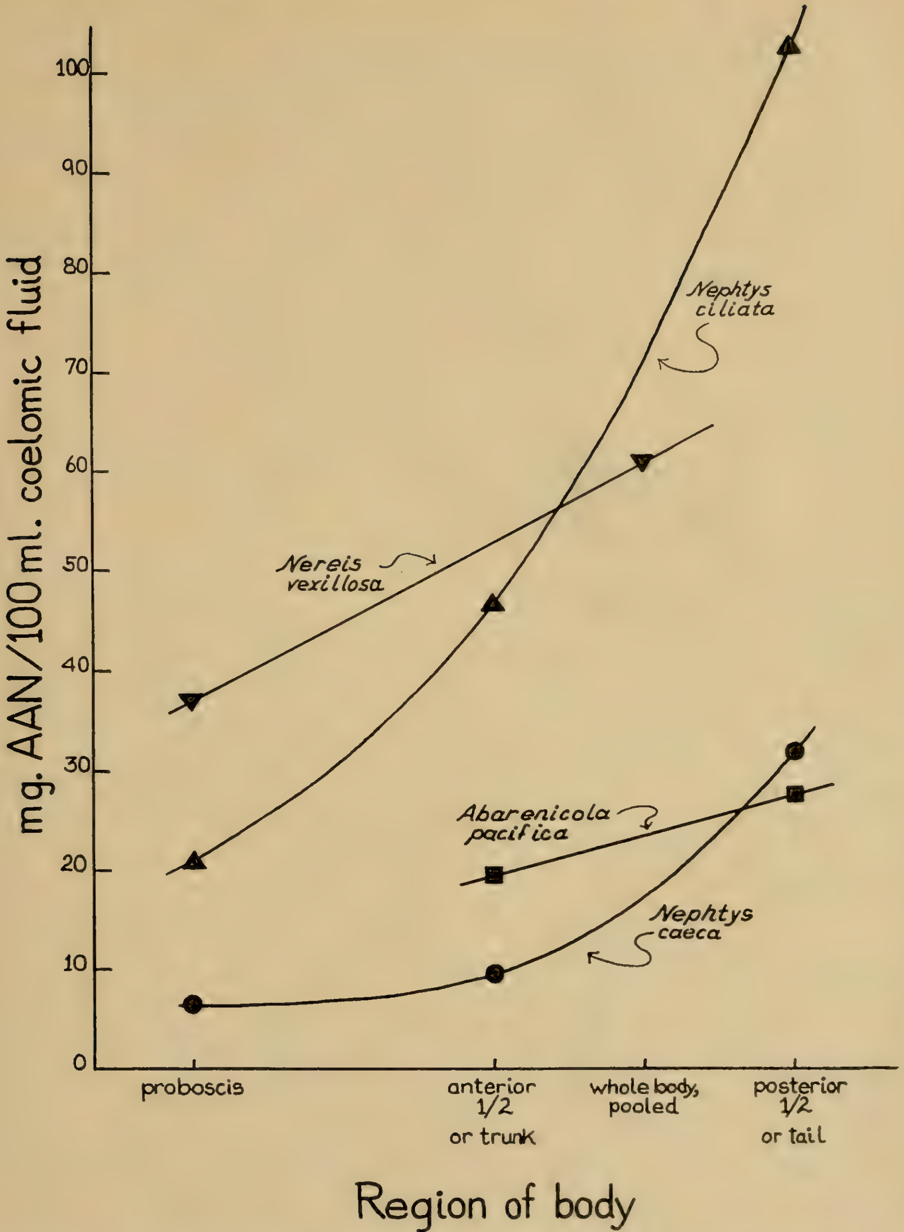

FIGURE 3. Amino-acid nitrogen (AAN) levels in coelomic fluid from different body regions.

sample contributed by different regions of the worm. In all four species examined for regional differences, a gradient in coelomic fluid AAN levels was found which increased posteriorly (Fig. 3). Other variations within species are hard to explain on the basis of the present data. Some possible factors will be discussed later.

TABLE II

Levels of ammonia nitrogen (NH_3 N) and amino-acid nitrogen (AAN) in the coelomic fluid and body wall of various polychaetes. Where several regions of the body have been sampled from the same group of worms, this is indicated by ditto marks in the column headed "Number of worms"

Species	No. worms	Body region	Coelomic Fluid		Body Wall	
			NH_3 N mg./100 ml.	AAN mg./100 ml.	NH_3 N mg./100 g. H_2O	AAN mg./100 g. H_2O
Nephtys caeca	2	proboscis	0.32	6.49		
	″	anterior 1/2	0.45	9.45		
	″	posterior 1/2	1.52	31.87		
Nephtys caecoides	10	whole	1.56	44.55	19.4	236.7
Nephtys ciliata	19	proboscis	0.50	30.64		
	″	all body	1.20	44.28		
	6	proboscis	tr?	20.93		
	″	anterior 1/2	1.40	46.80		
	″	posterior 1/2	2.80	102.48		
	5	whole	1.44	71.34	22.3	291.8
	4	whole	1.92	35.43	30.7	341.3
Nereis vexillosa	8	proboscis	tr?	37.35		
	″	all body	1.60	60.59		
	8	whole	tr?	19.68	5.6	463.4
	10	whole	0.83	18.13	3.6	486.0
	9	whole	—	—	4.2	491.2
Diopatra ornata	8	whole	1.60	19.87		
	25	whole	6.02	75.55		
Eunice biannulata	9	whole	2.07	66.72	23.1	330.7
	8	whole	1.23	72.60	45.5*	358.9
Nainereis laevigata	60	whole	4.80	75.84		
Amphitrite cirrata	3	thorax	0.38	9.22	6.2	242.7
	5	thorax	0.18	40.62	10.5	337.6
Neoamphitrite robusta	1	thorax	0.50	17.13		
	5	thorax	0.60	32.91	8.8	513.8
	7	thorax	0.40	22.61	15.7	587.8
Thelepus crispus	2	thorax	2.39	162.13	19.3	686.2
	5	thorax	1.17	150.63	2.1	516.3
Abarenicola marina	7	trunk	0.40	16.97		
	″	tail	0.60	27.70		
	4	trunk	0.84	5.50	62.3*	385.5
	4	trunk	0.83	18.42	5.9	337.3
Aphrodite japonica	1	whole	tr?	1.64		
	1	whole	0.05	0.50	15.4	186.3
Travisia pupa	1	whole	—		36.5	398.4
	1	whole	0.18	7.05	13.2	529.9

* High NH_3 N value because tissue left several hours at room temperature.

Amino-acid nitrogen levels vary considerably between species of polychaetes (Table II and Fig. 2). Subtidal forms such as *Aphrodite* and *Travisia* have very low levels, only a few milligrams per 100 ml. *Nephtys caeca,* which is subtidal also, has distinctly lower levels than the other species of *Nephtys* studied which are primarily intertidal in distribution. Among the intertidal forms, the nephtyids, nereids, eunicids, and the one species of orbiniid studied, *Nainereis laevigata,* fall within the approximate range of 20–80 mg. AAN/100 ml., and *Abarenicola* between 5 and 30 mg. AAN/100 ml. A similar range is found in two of the three terebellids studied, *Neoamphitrite* and *Amphitrite.* It is of interest, therefore, that *Thelepus* normally has very high levels, 150 mg. AAN/100 ml. This species was collected side-by-side on the same beach (often attached to the same rock) as the other two terebellids, the only apparent difference in habitat being that *Thelepus* secretes an extremely tough tube, while those of the other two species are thick, but of friable sandy construction. In the laboratory, the animals were maintained without tubes for several days prior to sacrificing them, so the differences are not likely to be dependent on differences in permeability of the tubes, which could conceivably permit regulation of the intra-tubular micro-environment.

At first it was thought possible that the high AAN of *Thelepus* coelomic fluid was due to partial hemolysis of the numerous blood cells characteristically found in terebellids, and in fact, in a preliminary experiment when uncentrifuged coelomic fluid from *Amphitrite* was frozen and thawed prior to making an alcoholic extract a value of about 140 mg. AAN/100 ml. was obtained from that species. However, great care was taken in handling the samples from all three species of terebellids to avoid hemolysis. Moreover, in osmotic stress experiments at reduced salinity (Clark, 1968), it was found that the AAN values of *Thelepus* coelomic fluid fall to a much lower value (about 40 mg./100 ml.) although there was no obvious fall in "hematocrit" and measurements on body wall showed a level of intracellular AAN still well over 400 mg./100 g. tissue water. The extremely high coelomic fluid value in *Thelepus* thus appears established, and is an interesting anomaly.

DISCUSSION

In the coelomic fluid of *Nephtys hombergi* about 75% of Nin + N is due to free amino acids (excluding taurine, which was not measured) (Clark, 1964). It seems probable, in the light of our knowledge of intracellular free amino acids in polychaetes (Awapara, 1962), that free amino acids and taurine form the bulk of the α-amino nitrogen found in the coelomic fluid and body wall of other species studied here, with oligopeptides possibly contributing a small proportion, as they do in *Nephtys hombergi* coelomic fluid (Clark, 1964). The ninhydrin method gives reasonably good estimates of α-amino nitrogen, including taurine, and of ammonia, but does not include the prolines. Hydroxyproline has not been reported as a free amino acid among invertebrates so far studied. Proline constitutes about 5% of the AAN of *Nephtys hombergi* coelomic fluid (Clark, 1964) and accounts for from 10 to 20% of the amino acids in the body wall of nereids (Jeuniaux *et al.*, 1961) although it is absent from *Arenicola marina* (Duchâteau-Bosson *et al.*, 1961). Thus the AAN values reported here are likely to be somewhat lower in some species than if the proline values were included, but it is unlikely that its exclusion significantly alters the comparative results. In addition, the body-wall estimations are likely to be too low because the water volume on which they are based includes an unmeasured proportion of extra-cellular water, which is presumably in equilibrium with the coelomic fluid in respect to low molecular weight constituents. Its volume, which may vary from species to species, is unknown. The inulin space in molluscan muscle varies from about 10 to 25% (Potts, 1958; Bricteux-Grégoire *et al.*, 1964a, 1964b). The true intracellular AAN and NH_3 N levels are therefore higher than reported.

Amino-acid levels within a given species tend to be somewhat variable, and this is particularly true of coelomic fluid estimates. The better agreement obtained with body-wall samples, particularly those of *Nereis vexillosa,* suggests that the experimental procedures *per se* permit reproducible results. Some possible causes of intraspecific variation may be considered.

Since precautions were taken to prevent diffusion of amino acids from coelomocytes from contaminating coelomic fluid samples, it seems unlikely that this was a major cause of the variation. Contamination with blood is another possibility. In terebellids, it is difficult to observe whether blood has contaminated the sample due to the opacity of the coelomic fluid. Centrifuged supernatants varied in the amount of red color present, partly due to blood pigments. In the eunicids the relatively small volume of the coelom makes it difficult to obtain sufficiently large samples, and the extensive internal capillaries associated with the branchiae are unavoidably damaged during collection of fluid. Although Bahl (1947) found in the earthworm, *Pheretima posthuma,* an equal distribution of urea between blood and coelomic fluid, but slightly higher levels of other NPN constituents in the blood, the total levels are so low (amino acid = 6 mg./100 ml. blood) as to offer little clue to the condition in polychaetes. The numerous thin-walled capillaries in polychaete vascular systems suggest that equilibrium of low molecular weight components between blood and coelomic fluid is likely, as is the case for non-protein nitrogen constituents between the blood and lymph in mammals (Peters and van Slyke, 1946).

Previous work on *Nephtys* led to the prediction of biochemical gradients along the length of regenerating animals (Clark, 1965). Data obtained subsequently on both intact and regenerating *Nephtys hombergi* (see Table I for values for intact worms) confirmed the existence of pH gradients, though in the opposite direction to that postulated. When the same animals are sampled from various regions of the body the pH is usually found to decrease in a posterior direction (Fig. 1), while levels of ninhydrin-positive substances increase (Fig. 3). It seems likely that such gradients could be a major factor in the variability of AAN levels in coelomic fluid samples within a species. It would be of interest to know whether the observed gradients are due to metabolic gradients in the tissues, or are due to as yet undetected disproportionalities in the functional levels of different tissues along the length of the body, particularly in those species in which all the segments appear so uniformly similar. In particular, gradients in nephridial function seem a possible explanation.

It is also possible that intraspecific coelomic-fluid AAN variability is due to other factors such as starvation or degree of maturation, although neither of these appears to influence the amino-acidemia of *Nephtys hombergi.* It is of interest, however, that mature *N. hombergi* have a higher non-ninhydrin-positive NPN than do immature worms (Clark, 1964). A possible factor affecting coelomic fluid AAN is the micro-environment provided by the construction of a more or less impermeable tube. Most of the animals studied were maintained without tubes, but worms of one batch of *Diopatra,* in which there was a high coelomic fluid AAN, were allowed to remain in their tubes. Although the data are insufficient for a conclusion, it seems possible that animals in semi-permeable tubes lose less of their excreted nitrogen to the ambient sea water, and may thus establish a more favorable concentration gradient for maintaining a high level of amino acids in the coelomic fluid.

The last and most likely explanation is that amino-acid levels in polychaetes are not closely regulated and vary within a fairly wide range.

There is as yet no explanation for the high amino-acidemia now found in several different species of intertidal polychaetes, and such high amino-acidemias were once thought to be diagnostic of the Insecta (Florkin, 1959). The enormous range of AAN exhibited among species of polychaetes suggests they are biochemically a very diverse group, an idea supported by the great variety of phosphagens (Ennor and Morrison, 1958) and phosphokinases (Virden and Watts, 1964) also found in this class.

The role of the high amino-acidemias in those polychaetes where they occur is not clear. They may act as an osmotic buffer or serve as a reservoir for the intracellular free amino-acid pools, but it is hard to understand why they are nearly absent from some polychaetes and from certain other euryhaline marine invertebrates with high tissue levels of amino acids (Camien *et al.*, 1951; Stevens *et al.*, 1961).

It is also difficult to explain the function of the high levels of free amino acids in tissues of invertebrates. The ability of many soft-bodied invertebrates, including several polychaetes, to take up free amino acids from sea water across the body wall against a very unfavorable concentration gradient is well-known (Stephens, 1963, 1964; Stephens and Schinske, 1961; Stephens and Virkar, 1966; Virkar, 1963, 1965), and suggests that a high proportion of the nutritional requirements of these animals could be met this way. On the other hand, the maintenance of high levels despite starvation suggests that some other role is also involved. As pointed out by Shaw (1958), the primary role of intracellular free amino acids cannot be osmoregulatory since high concentrations occur in the tissues of stenohaline animals like *Homarus*. This would apply also to a subtidal polychaete like *Travisia* which is probably never normally subjected to major osmotic dilution, yet has a very high intracellular AAN. The possibility exists that the substitution of inorganic ions by amino acids intracellularly serves another function, such as chelating toxic heavy metal ions found in sea water (Tyler and Rothschild, 1951) or buffering intracellular pH.

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SUMMARY

1. Coelomic fluid amino-acid nitrogen (AAN) shows great variability among the fourteen polychaete species examined, ranging from about 1 to 5 mg./100 ml. in subtidal species to more than 150 mg./100 ml. (about 100 mM/l.) in an intertidal species. The concentration of ammonia nitrogen (NH_3 N) in coelomic fluid is roughly about 5% of the AAN.

2. Individual variation in coelomic fluid AAN normally occurs intraspecifically and possible sources of variability are considered.

3. Axial gradients exist in all species examined for this. Amino-acid concentration always increases posteriorly, and pH usually decreases in the same direction.

4. Intracellular (body-wall) AAN is relatively less variable both within and between species, the range being from about 130 mM/kg. tissue water in *Aphrodite* to about 400 mM/kg. in two terebellids. Concentrations do not appear to correlate with habitat. Tissue NH_3 N levels are slightly higher proportionately than in coelomic fluid, and are more variable.

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FACTORS CONTROLLING EMERGENCE OF PINK SHRIMP (PENAEUS DUORARUM) FROM THE SUBSTRATE[1]

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Diurnal patterns of activity occur in many species of penaeid shrimp. *Penaeus duorarum* Burkenroad, like most species, is active at night but remains burrowed beneath the sand during the day (Idyll, 1950; Williams, 1955, 1958; Viosca, 1957; Eldred, 1958; Iversen and Idyll, 1959; Eldred *et al.*, 1961; Fuss, 1964; Fuss and Ogren, 1966; and Wickham, 1966). Most of these records have been derived from incidental observations and little is known of the nature of the control of this periodicity in behavior.

Fishermen have long been aware that dull weather or murky waters can increase their catches during the day. Conversely an increase of light intensity at night has been shown to cause reburrowing of shrimp which are already above sand (Eldred *et al.*, 1961). Fuss and Ogren (1966) stressed the influence of light on the daily activity patterns but also found "some evidence of an endogenous component." The presence of this endogenous component was clearly shown by Wickham (1966) who found that a rhythm controlling the times of activity would persist noticeably for longer than a week, under apparently uniform external conditions.

This study concentrates on one aspect of the diurnal activity pattern, the initial emergence from the sand at dusk. This activity is markedly synchronized in all members of the population. An attempt is made here to determine the factors eliciting emergence and maintaining its synchrony in all individuals.

METHODS

The shrimp used in the experiments were all juveniles and subadults and were housed in twelve 15-gallon aquarium tanks with a sand substrate 6–8 cm. deep. The water, maintained at a depth of approximately 20 cm., was in constant circulation and was aerated through subsand filters.

The tanks were housed in a light-proof enclosure. The shrimp were exposed either to natural day-night cycles, by uncovering the window against which the tanks were placed; or to artificial cycles of light and dark, by the use of a time switch connected to a 150w flood-lamp. The flood-light was diffused by deflecting it off the white roof of the enclosure. The light intensity incident on the surface of the substrate during the light period was approximately 13 lux. During the dark period the enclosure was illuminated by means of a low intensity red light.

The shrimp were netted from a canal in the Everglades of southern Florida. Six to ten shrimp (3–12 cm. total length) were housed in each tank and fed on small pieces of fish (mullet). Virtually no mortality occurred and, unless the water became polluted or the circulation and aeration mechanisms failed, the shrimp showed no signs of "distress."

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[1] Contribution No. 856 from the Institute of Marine Sciences, University of Miami.

RESULTS

Diurnal pattern of burrowing behavior

The activity of this species shows a clear phase relationship with the day-night cycle. Figure 1A shows the typical pattern of burrowing behavior of 50 shrimp recorded on three consecutive days when exposed to a natural day-night cycle.

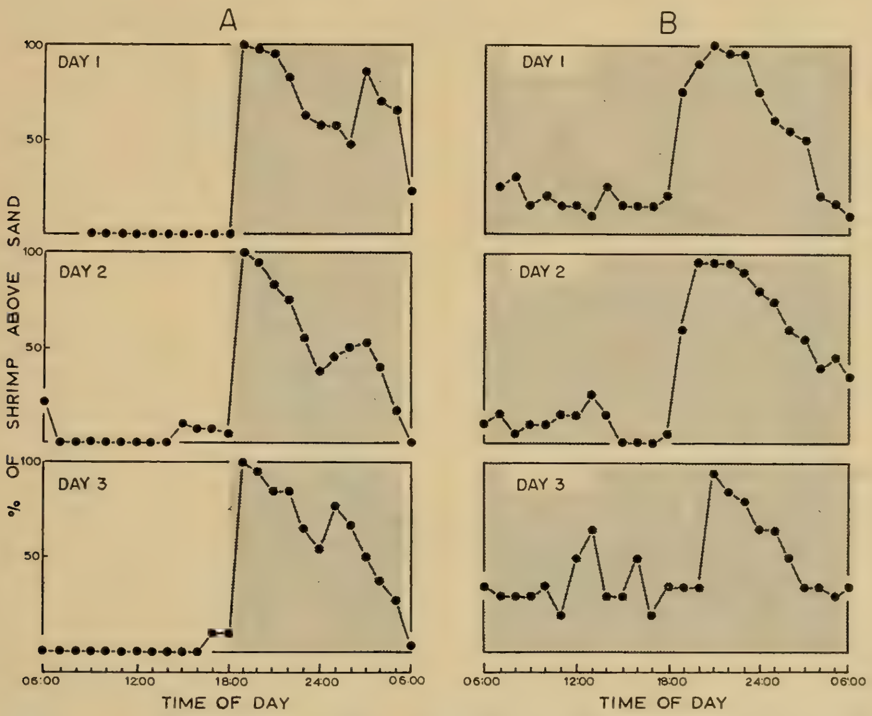

FIGURE 1. The burrowing of shrimp during three consecutive days when exposed to (A) a natural day-night cycle and (B) constant low-intensity illumination. Ten shrimp were housed in each of five tanks: counts of the number of shrimp above sand were taken at hourly intervals.

The shrimp are active above the substrate at night but are inactive and buried during the day. The close association between sunset and the onset of activity (in this case emergence) confirms the records of Fuss and Ogren (1966) and Wickham (1966). These workers also reported a two-peaked pattern of occurrence above sand at night, similar to that shown in the figure.

The persistence and circadian periodicity of this pattern under conditions of low light intensity (Wickham, 1966) is confirmed by Figure 1B. The pattern is shown here for only three consecutive days, but, as shown by Wickham (1966), the evening "peak" of emergence was still clearly noticeable after one week and the free-running period throughout this time was clearly longer than 24 hours.

These results suggest that a circadian rhythm in phase with the light-dark cycle is responsible for the pattern of occurrence above sand.

Emergence at the time of the light-dark transition

An indication of the degree of synchronization existing between all individuals is given by Figure 2. When exposed to a natural day-night cycle almost all shrimp would, at the time of sunset, emerge from the substrate within a period of 20–30 minutes. The results shown are for days on which there was no cloud cover and consequently on which there was a relatively rapid decrease of light intensity at the time of sunset.

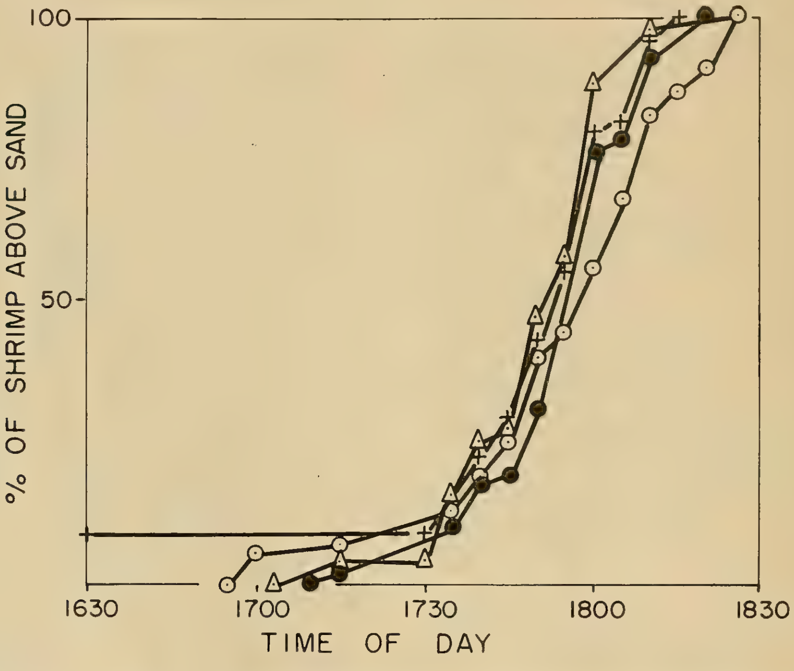

FIGURE 2. The patterns of emergence at sunset on four consecutive days of approximately 75 pink shrimp exposed to a natural day-night cycle. Counts of the number of shrimp above sand were taken at approximately 5–10-minute intervals.

Preliminary observations indicated that smaller shrimp generally emerged before larger individuals. To test this possibility, the time of emergence of every shrimp was recorded on eight consecutive evenings. Figure 3 shows the mean time of emergence of each of 8 size categories and clearly indicates that emergence of small shrimp (2–3 and 3–4 cm. categories) occurred approximately 18 and 12 minutes, respectively, before those in the larger size categories (4–5 cm. and larger). Although these results suggest that a progressively later mean time of emergence occurs with increase in size above 4 cm. this is not clearly evident.

Figure 4 shows that the time of burrowing at dawn is again different in different size groups: larger shrimp burrow earlier than smaller individuals.

A possible explanation for this is discussed later.

The close association between emergence and the time of the light-dark transition suggests that it is the light-dark transition which is a factor maintaining synchrony between the circadian rhythm and the light-dark cycle (*i.e.*, it is the Zeitgeber).

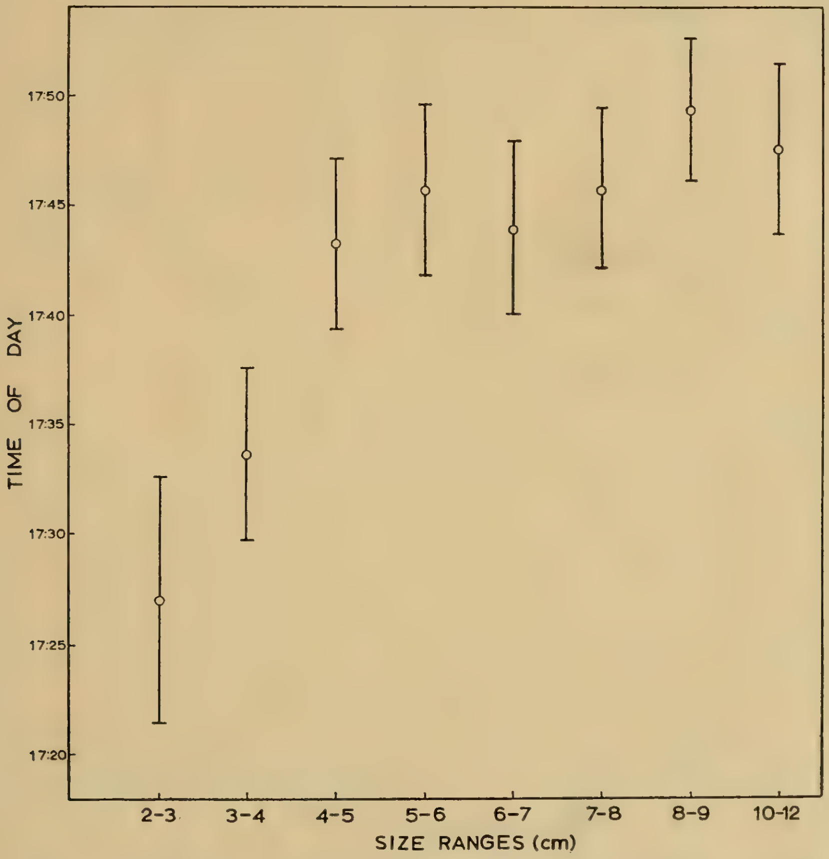

FIGURE 3. The mean time of emergence of each of 8 size ranges of shrimp. The time of emergence of each individual was recorded for 7 days. One shrimp of each size category was housed in each of 10 tanks. The points therefore represent the mean time of emergence of 70 shrimp. The vertical bars represent plus or minus one standard deviation.

If this is the case, a "periodically changing sensitivity of the organism to the stimuli of the Zeitgeber" should be apparent (Aschoff, 1965, p. 95). To determine this, emergence, as a response to light-dark transitions imposed at various times (09:00, 12:00, 15:00 and 18:00 hrs.), was recorded (Fig. 5). Prior to recording emergence all the shrimp (75–100) were maintained under an artificial light cycle (LD

12:12) for at least ten days. This cycle coincided closely with the natural cycle. The time of the light-dark transition was 18:00 hrs. and, like all the transitions, was abruptly imposed.

The patterns of emergence, compared here and in later experiments, were markedly consistent for different stocks of shrimp maintained under identical light-dark cycles, and temperatures, and similarly fed. These patterns can therefore be used as reliable indices of the influence of all the stimuli causing emergence under the particular set of conditions.

Emergence as a response to a light-dark transition imposed at 18:00 hrs. was relatively rapid (Fig. 5). At temperatures of approximately 25° C., 90% or more emerged within the first 30 minutes of the light-dark transition. Following emergence, the shrimp carried out swimming and food-searching movements and seldom reburrowed until several hours later, at the time of the quiescent period between the peaks of activity.

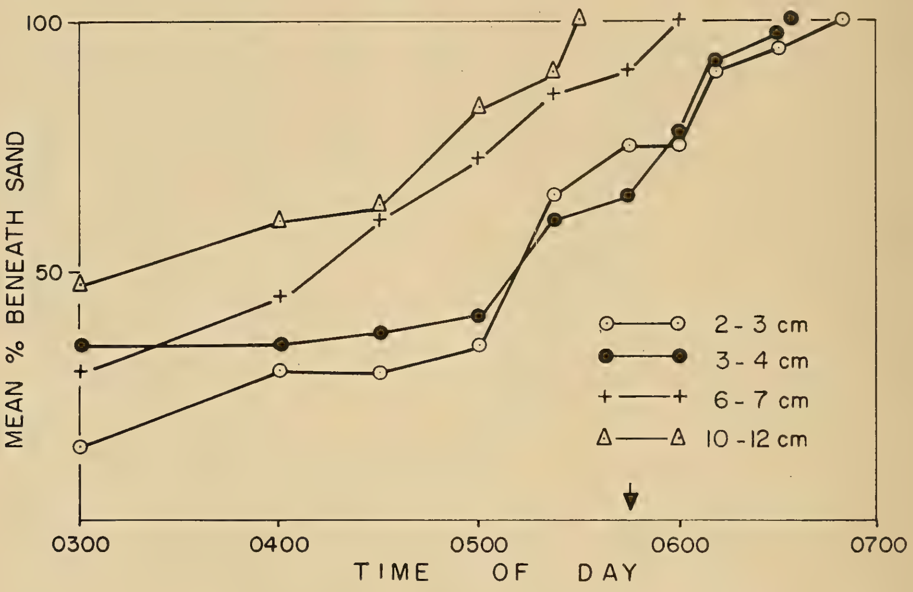

FIGURE 4. Burrowing at dawn of 4 size categories (10 shrimp in each category). Each point represents the mean percentage beneath sand for 3 consecutive days.

When the light-dark transition was imposed at a time other than at 18:00 (*i.e.,* at 09:00, 12:00 or 15:00 hrs.) emergence occurred more slowly. Seldom more than 70% emerged within 90 minutes. Those that did were seldom active and a large proportion would reburrow within an hour.

This is clear evidence for a changing responsiveness to the light-dark transitions and implies that the light-dark transition is the Zeitgeber, or an important component of it, which maintains the synchrony between the internal oscillation of the rhythm and the external light-dark cycle.

Phase-shifting of the light-dark cycle

To elucidate further the nature of factors responsible for phase synchronization of rhythm with environment, experiments involving shifts of the phase of the light-dark cycle were conducted.

The response of an observed rhythm to a shifted Zeitgeber has been commonly employed as an indication of phase dependence of the rhythm on a particular environmental factor (for a review of the literature on entrainment see Aschoff, 1963). Aschoff (1960) stated (p. 20) that "if the Zeitgeber is fully effective, the organism should follow the shift within a few periods and should regain the original phase-relation to the Zeitgeber."

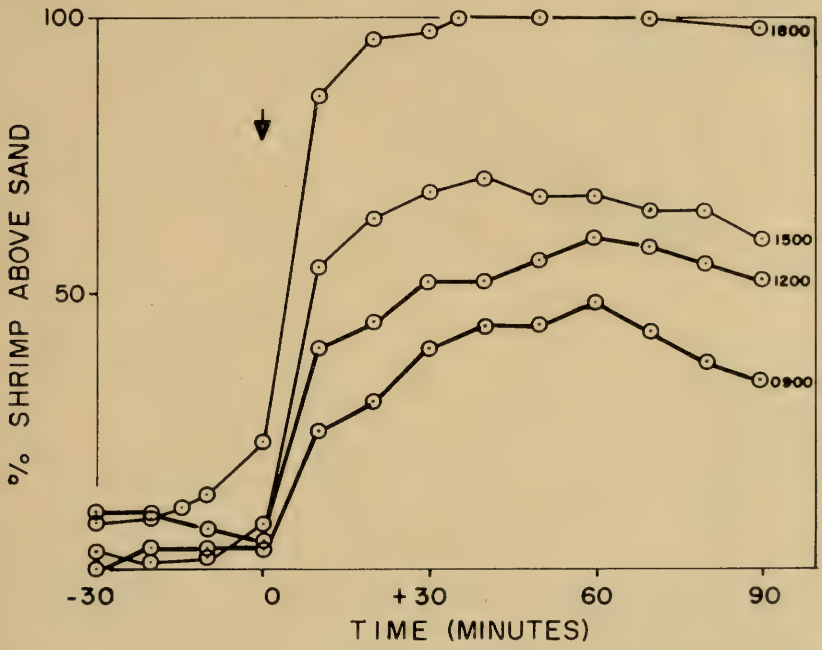

FIGURE 5. The pattern of emergence of shrimp when exposed to light-dark transitions imposed at 09:00, 12:00, 15:00 and 18:00 hours. The shrimp had been entrained to a LD 12:12 cycle in which the light-dark transition occurred at 18:00 hours. The points shown for 09:00, 12:00 and 15:00 hrs. are in each case the mean of two day's records while those for 18:00 hrs. are the mean of three.

In the following experiments the phase of an LD 12:12 light cycle was altered through an angle of 120° such that the light-dark transition occurred at 10:00 hrs. instead of 18:00 hrs. The pattern of emergence at the time of the light-dark transition of the altered regime was recorded for several days following the phase-shift of the light-dark cycle (Fig. 6). Two equal-sized groups, each comprising 40 individuals, were compared. From the day on which the light-dark cycle was first altered the shrimp in group A were fed following their emergence, *i.e.*, on completion of the records of emergence at approximately 11:30 hrs. Group B was not fed at all after the cycle had been shifted. The records were continued until emergence was once more maximal (as at 18:00 hrs., prior to the phase-shift) and until subsequent activity occurred without reburrowing. At this stage the circadian rhythm was considered to have returned to its prior phase relationship with the shifted Zeitgeber.

Emergence of group A after exposure to only one cycle of the altered light regime was almost maximal. An approximately equivalent pattern of emergence was only achieved by group B, following a series of transient stages, after four days exposure to the altered cycle. It appeared, however, that group A did not achieve complete resynchronization with the shifted light-dark cycle after only one day for, despite the almost maximal emergence, there still remained the tendency of some shrimp to reburrow after emergence. (This is presupposing that emergence and subsequent activity are both responses to the same oscillating system.) By the third day, however, the pattern of emergence and subsequent activity of Group A could not be distinguished from that which occurred in shrimp long exposed to a particular light-dark cycle, *e.g.*, Figure 5, 18:00 hrs. Group B by comparison required six days to reach an equivalent pattern.

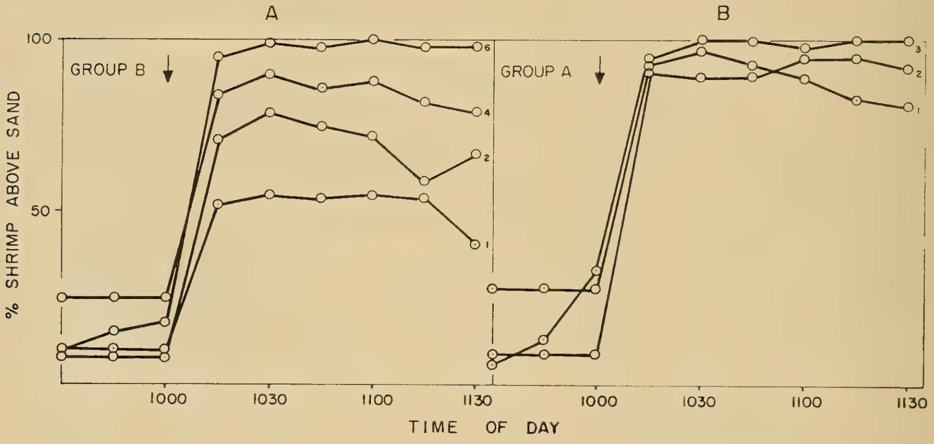

FIGURE 6. The resynchronization of the phase of the rhythm controlling emergence to a shift (120°) in the phase of the light-dark cycle in (A) shrimp which were *not* fed throughout the time of re-entrainment, and (B) shrimp which were fed each day immediately following the light-dark transition of the "new" cycle. The numbers at the end of each emergence pattern indicate the number of days since the shift was imposed.

Further evidence that resynchronization had occurred in both groups of shrimp was derived from experiments which showed that approximately 40% of the shrimp would emerge at and following the time of the light-dark transition of the altered cycle, even when *no* transition was imposed.

Experiments were conducted to determine whether the slower resynchronization of the rhythm with the light-dark cycle which occurred in group B (unfed) compared with group A (fed) was due to starvation with perhaps a consequent depression of activity.

The effects of feeding and starvation on emergence

The pattern of emergence of a group of 40 shrimp which had not been fed for four days was compared with that of an equivalent group, maintained under identical conditions but which were fed each day after the onset of the dark period. For the purposes of this experiment emergence was recorded when a light-dark transition was imposed at a time (10:00 hrs.) out of phase with the time to which they had been entrained to emerge (18:00 hrs.).

The results (not detailed here) showed that in two out of three repetitions the rate and degree of emergence of the unfed shrimp were greater than those of the fed shrimp. On the third occasion there was no noticeable difference between the two groups. These results suggest that the difference in the time taken to resynchronize the rhythms of the fed and the unfed shrimp, following phase shifting of the external light cycle, was not a result of depression of activity caused by starvation.

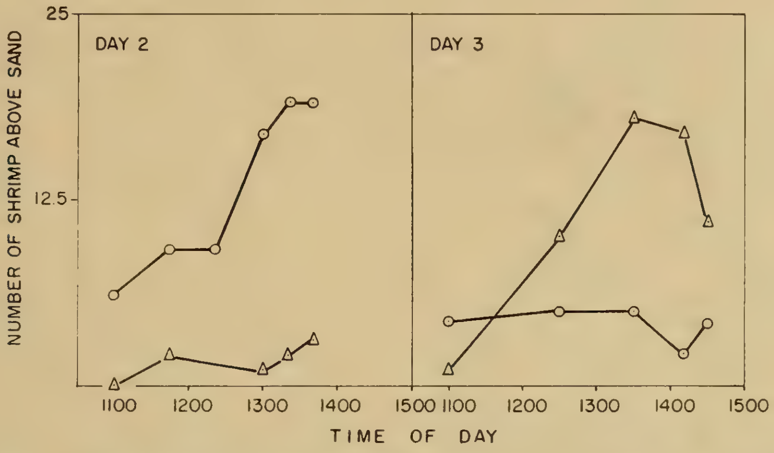

FIGURE 7. The emergence during the day of two groups of shrimp (A and B) subjected to different feeding schedules. On day 1, group A (○———○) was fed during the light period (13:00 hrs.); while group B (△———△) was fed immediately after the light-dark transition (19:45 hrs.). Consequent emergence patterns are shown for day 2. Later on day 2, group A was fed at 19:45 and group B at 13:40—*i.e.*, the times of feeding were reversed. Their emergence is shown for the following day, day 3. (Note: shrimp were deprived of food for 3 days before day 1.)

Evidence for a feeding rhythm

Two groups (A and B), each comprising 30 shrimp, were used in this experiment. They were both held under identical conditions (LD $13\frac{1}{2}$; $10\frac{1}{2}$) but were fed at different times. To separate emergence, which could be the result of a feeding rhythm, from normal nocturnal emergence and activity, one group was fed at midday while the other was fed after their emergence in the evening (19:45). (Under normal circumstances the shrimp do not emerge during the day, but when not satiated they can be induced to emerge if food is placed in the tanks.) The emergence of both groups was then recorded the following day. The results (Fig. 7) show that whereas the group (A), which had been fed at midday of the previous day, emerged in fair numbers approximately 24 hours after being fed, very few of group B which had been fed at 19:45 the previous day were above sand at the same time. When group B was fed at 13:40, and group A at 19:45 the position was immediately reversed: whereas group B emerged in numbers 24 hours later, group A largely remained beneath the substrate.

These results will be discussed below.

Discussion

The time of activity of this species shows a very clear phase-relationship with the day-night cycle. The most marked manifestation of this association is the close relationship existing between the time of emergence from the sand and the time of the light-dark transition of the day-night cycle. Emergence of almost the entire population takes place within a period of 20–30 minutes at about the time of sunset. The findings of this study concern the mechanisms whereby this synchrony of emergence between all members of the population is maintained.

Two rhythms are shown to be involved: (i) a circadian rhythm in phase with the light-dark cycle, and (ii) a 24-hour feeding rhythm. Experiments were designed to distinguish the influence of each and they are discussed separately below.

(i) Circadian rhythm of emergence from the sand

The dependence of the phase of the rhythm on the light-dark cycle was shown by experiments in which the latter was shifted through an angle of 120°. Six days of entrainment to the altered cycle were required to resynchronize the rhythm in shrimp which were not fed following the shift of cycle. (The fact that these shrimp were deprived of food throughout the re-entrainment process indicates that the basis of this rhythm is independent of the 24-hour feeding rhythm, discussed below.) This is in accordance with Aschoff's (1963) findings which showed that in general many organisms need three to six days for a 6-hour phase shift, *i.e.*, one to two hours shift per day.

An indication of the influence of the rhythm is further shown by the fact that after phase synchrony had been achieved in the above experiment, emergence of about 40% of the population occurred at the time of the altered light-dark transition even when no transition was imposed and despite the prevailing high light intensities which usually inhibit emergence.

Several factors indicated that the light-dark transition is the Zeitgeber, or an important component of the Zeitgeber, responsible for the synchronization of the circadian oscillations with the period of the day-night regime. As mentioned above, a very close association existed between emergence and the light-dark transition and, when the time of this transition was shifted in either direction by about an hour, it was always followed by a shift in the mean time of emergence.

A changing responsiveness to a light-dark transition was indicated by experiments in which emergence was recorded at 09:00, 12:00, 15:00 and 18:00 hours. Emergence which occurred as a response to a light-dark transition at the time when the shrimp had been entrained to receive a transition (18:00 hrs.) elicited emergence which occurred rapidly (90% within 30 minutes). Emergence at any other time was always very much slower; fewer shrimp emerged and those that did frequently reburrowed shortly afterwards. This is clear evidence that the light-dark transition is important as the Zeitgeber (Aschoff, 1965).

(ii) 24-hour feeding rhythm

The time taken to resynchronize the phase of the rhythm with a phase shift in the light-dark cycle was considerably shorter in shrimp which were fed following the light-dark transition than in those unfed during the period of re-entrainment. A feeding rhythm with an approximate 24-hour periodicity probably accounts for this difference. This rhythm was indicated by experiments in which shrimp, induced to emerge to feed during the day, would again emerge and carry out food-searching movements at approximately the same time the following day when *no* food was present.

The strength of the response is shown by the fact that emergence took place despite the inhibitive factor of the high light intensity.

The results suggest the existence of an endogenous "time sense" similar to that first described in bees by Forel (1910) and later by v. Buffel-Reepen (1900), Beling (1929), Wahl (1932) and reviewed more recently by Renner (1960).

Individuals smaller than 4 cm. (total length) emerged appreciably earlier than those larger than this size. At dawn the large individuals would generally reburrow into the substrate some time before those in the smaller size ranges. It is probable that the time of activity of smaller individuals is regulated more by the exogenous factors of change in light intensity than by some inherent rhythmic process. This contention appears to be supported by the pattern of dawn burrowing in which a marked increase in rate of burrowing of smaller individuals was shown at about the time that light intensity increases most suddenly. The pattern of burrowinging of larger individuals showed no marked increase at this time.

Experiments in which the emergence of both fed shrimp and shrimp deprived of food for several days was compared, and in which a light-dark transition was imposed at a time out of phase with the entrained light-dark transition, indicated that food deprivation did not lead to a decreased rate or degree of emergence. On the contrary, the results suggested that shrimp deprived of food emerged more rapidly than fed shrimp. A possible explanation for this could be that food deprivation, by affecting metabolism directly, or by causing the shrimp to emerge during the day to search for food, "breaks down" the circadian activity rhythm and/or the feeding rhythm, thereby rendering the shrimp more susceptible to exogenous stimuli.

The possible adaptive significance of synchrony of emergence

The nocturnal activity of shrimp and the close association of its onset with the light-dark transition has a number of possible advantages. The observations of Fuss and Ogren (1966) have shown that shrimp active above the substrate by day readily fall prey to many species of fish. A great measure of protection from predators is acquired, therefore, by the limitation of the time of activity to the time of darkness.

In addition there is evidence (Hughes, 1967) that all activities occurring at night are temporally related to the time of onset of activity at dusk and therefore this initial synchrony will considerably influence the synchrony of subsequent activities. It is therefore possible that maintenance of cohesion of aggregations of shrimp is largely maintained by rhythmic control over most activities, and their consequent synchrony in all individuals. Maintenance of these aggregations may have survival value with regard to predators and will be clearly advantageous at times of mating and spawning when groups at a similar developmental stage will arrive at suitable spawning sites together.

The apparent advantage of the circadian rhythm controlling emergence and possibly subsequent activity is clear, but that played by the 24-hour feeding rhythm is less clear. However, it is usual that the first activity following emergence is feeding. Thus the two rhythms will virtually be in phase and may serve to reinforce each other in such a way that emergence of all individuals becomes more closely synchronized.

I would like to express my appreciation for the generous financial assistance given by the National Geographic Society, Washington, D. C., who have sponsored this research project. I also thank Dr. C. P. Idyll, of the Institute of Marine Sciences, University of Miami, for providing facilities within the Division of Fishery Sciences.

SUMMARY

1. A close relationship exists between the day-night cycle and the times of activity of pink shrimp. The shrimp bury beneath the substrate during the day but emerge at the time of sunset and are active at night.

2. Emergence from the substrate is markedly synchronized in all members of the population. This study elucidates the mechanisms whereby this synchrony is maintained.

3. The persistence of the pattern of nocturnal activity for several days under conditions of constant low light intensity confirmed that emergence and subsequent activity were under rhythmic control.

4. Resynchronization of the phase of the rhythm controlling emergence to a shift in the light-dark cycle indicated that the light-dark cycle itself, or some component of it, is responsible for maintaining the relationship between emergence and the day-night regime.

5. The close association between emergence and the time of light-dark transition indicated the possibility that the latter was the important component of the Zeitgeber responsible for the control of the time of emergence. This was supported by experiments in which a changing responsiveness was shown to light-dark transitions imposed at various times during the light period. Maximum response (in terms of the rate and degree of emergence from the substrate) occurred when a light-dark transition was imposed at a time (18:00) to which the shrimp had previously been entrained to receive the onset of the dark period.

6. Experiments in which shrimp, which had been fed 24 hours previously, emerged from the substrate and carried out food-searching movements, in the absence of food and despite high intensity illumination, which would normally suppress emergence, indicated the presence of a feeding rhythm with an approximate 24-hour periodicity.

7. The feeding rhythm probably accounts for the fact that the phase of the rhythm of emergence was resynchronized to a shifted light-dark cycle in three days among shrimp fed immediately following the light-dark transition of the shifted

cycle, as opposed to six days in those deprived of food during this time of entrainment. The phases of the two rhythms, being almost identical, probably supplement each other.

8. Shrimp smaller than 4 cm. (total length) emerged significantly earlier than larger individuals. This possibly reflects the lesser dependence of the early juveniles on inherent rhythms and consequently their greater receptivity to exogenous stimuli.

9. The most obvious advantage of the circadian rhythm controlling emergence and subsequent activity is to confine the times of activity of shrimp to the hours of darkness when predation by fish is minimal. The proximity of the phase of the 24-hour feeding rhythm to the circadian rhythm of emergence and activity probably serves to strengthen the entrainment properties of the latter such that the time of emergence is further synchronized between all members of the population. The synchrony of the time of emergence is probably important for the maintenance of cohesion between aggregations of shrimp.

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AIR-GAPING BY THE RIBBED MUSSEL, MODIOLUS DEMISSUS (DILLWYN): EFFECTS AND ADAPTIVE SIGNIFICANCE

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Pelecypods of the intertidal zone usually close their valves when exposed to air and open them when inundated. However, the ribbed mussel, *Modiolus demissus* (Dillwyn), does not follow this pattern. Rather, it leaves its valves ajar when exposed to the atmosphere during low tide (Kuenzler, 1961). This may be adaptive behavior associated with physiological responses by this organism to the high littoral habitat. This study investigates the pattern and effects of air-gaping by the ribbed mussel.

Air-gaping would be expected to be significant in aerial respiration and Kuenzler (1961) showed that this species respires in air at rates lower than those observed in water. Nevertheless, more oxygen is consumed in air than water since these mussels spend more time exposed than inundated. Kuenzler's study did not show how significant gaseous exchange is on aerial survival or why rates of respiration are lower in air for equal-sized mussels at the same temperature.

Bivalves have no cuticle and when gaping exposes the mantle cavity to air, rapid evaporation will desiccate them. Broekhuysen (1941) investigated rates of evaporation in 6 species of intertidal snails and found similar rates in all, but their intertidal position was correlated with ability to withstand desiccation. To investigate this problem in *Modiolus,* desiccation is studied as functions of mussel size, exposure time, and environmental temperature and humidity.

Transpiration does not have to be disadvantageous, for Edney (1957) showed evaporative cooling can be beneficial to intertidal isopods during high-temperature crises. This study attempts to find if evaporative cooling can depress the temperature of the ribbed mussel enough to significantly affect its high-temperature survival.

The ribbed mussel is abundant in the high intertidal zone and usually found half-embedded in the mud of salt marshes in clumps (*i.e.*, contagiously distributed) among the roots of *Spartina.* Observations by Lent (1964, unpublished) and McDougall (1943) indicate that mussels will also attach to surfaces more directly exposed to the atmosphere such as bridge and pier pilings. If air-gaping leads to desiccation when mussels are exposed, differences between the distribution patterns of mussels in moist marsh mud and those on exposed surfaces would be expected, and this study carefully examines these patterns.

This investigation then is an attempt to understand the significance of air-gaping by *Modiolus* to gaseous exchange, temperature and water balance, and intertidal distribution. It is a partial analysis of the physiological adaptations accomplished by this animal which enable it to succeed in a semi-terrestrial habitat.

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METHODS AND MATERIALS

Modiolus was collected from the Broadkill River near Bayside Laboratory of the University of Delaware at Lewes, Delaware, except where otherwise noted. Mussels were collected at low tide, taken to the Newark campus, scrubbed in tap water, placed in a natural sea water aquarium at 22 ± 2° C., and held a minimum of 24 hours. Mussels remained tightly closed during cleaning and the salinity was 28–30‰.

Shell height (the distance from the apex to the posterior margin) was measured to the nearest 0.1 mm. with vernier calipers. Lent (1967) showed that shell height is equivalent to size since an isometric relationship exists between shell height and length and an allometric relationship exists between shell height and all weight fractions.

Data on the air-gaping pattern were collected during July, 1965, when shell movements of 4 mussels were recorded during tides of varying duration. Tides were simulated by adding or removing water from a 6-gallon Nalgene carboy which floated in a sea water aquarium, and displacement altered the sea water level. One valve of the mussel was affixed to a solid substrate and the movable valve was connected to a mechano-electric transducer which pivoted on a ball-bearing fulcrum to yield a 3:1 step-down ratio. The step-down reduced the amplitude of shell movements below the limits of the transducer. Movements were recorded on a Grass Model 5D Polygraph. Mussels ranged from 22 to 78 mm. and the tidal period varied from 0.5 to 24 hours.

During July, 1965, 3 groups of 24 mussels (15–60 mm.) were placed in separate desiccators. The interiors were kept at 100% relative humidity by placing water in the bottom below a screen on which the mussels were located. The gaseous environments were air, oxygen and nitrogen. Nitrogen and oxygen were added by flushing for 3 minutes. Median survival time was the time when the 12th animal began to gape widely.

To measure mantle cavity oxygen tension, a 15-mm. hole was drilled through the right valve of several 65–85-mm. mussels. The hole accommodated the sensing electrode of a Beckman Model 777 Laboratory Oxygen Analyzer. A similar-sized hole was placed in the underlying mantle so ciliary currents could move mantle cavity water past the tip of the electrode. After drilling and cutting, mussels were replaced in the aquarium to insure responses had not been affected. The right valve was then secured, the electrode was inserted and sealed with Plasticene. Each mussel was inundated in a liter beaker of well-aerated sea water which was lowered and raised simulating tides. A total of 12 cycles was observed on 3 mussels. Mantle cavity oxygen tensions were compared to those of sea water at the beginning and end of each experiment.

Two desiccation experiments were performed. During November, 1964, 44 mussels (10–74 mm.) were exposed to air of 24.5 ± 0.5° C. and 80 ± 5% relative humidity (r.h.). Mussels were measured, weighed on an analytical balance (±1 mg.), and placed in filter paper cones with anterior end down. They were reweighed 7 times after 6¼, 17¾, 24½, 49, 74½, 100½, and 121½ hours. After the 24½-hour weighing, some were examined for signs of life. These were removed from the experiment, placed in individual beakers of sea water for 24 hours, and inspected for filtering activity, feces, pseudofeces, excreta, or fresh byssal threads.

The second experiment during June, 1965, measured the effect of temperature and humidity on desiccation rate. Eighty mussels (20–85 mm.) were taken from Canary Creek Bridge, Lewes, Delaware, and divided into 4 groups. Each group was exposed to 1 of 4 environmental conditions: 2 temperatures, 24 and 29 ± 1° C. and 2 humidities, 75 ± 10 and 100%. Mussels were weighed 6 times: at 0, $5\frac{1}{2}$, $17\frac{1}{4}$, $28\frac{1}{4}$, $48\frac{1}{2}$, and 68 hours. All animals were kept in the experiment until the last weighing and then tested for signs of life.

The procedure for measuring internal temperature is essentially the same as for mantle cavity oxygen tension. A 7-mm. hole was used, and the mantle was not damaged. The thermistor of a Yellow Springs Model 147 Scanning Telethermometer was inserted into the pallial cavity, the sea water level was lowered, and internal temperature was noted periodically for 24 hours. This procedure was executed 6 times with 3 individuals during June, 1966.

To test the effect of evaporative cooling on thermal survival, 5 groups of 20 mussels (24–87 mm.) were exposed to 5 temperatures in a Sherer Controlled Environment Lab Model Cel 37-14. The temperatures were 32.0, 35.0, 38.0, 40.5, and 43.0 ± 0.5° C., and relative humidity was 80 ± 5%. The stress lasted 10 hours. Half of each group was allowed to gape while the remaining 10 were clamped to prevent gaping and evaporation. After the stress, each mussel was opened and inspected for signs of life such as foot motility, mantle responsiveness, and ciliary activity.

During August, 1964, 700 mussels were collected from the areas formed by the angles between the bulkhead and pilings of Canary Creek Bridge. In 19 areas, all mussels were removed. Individuals were removed, their vertical distance in feet below the cross members of the bridge was measured, and their size was measured. The cross members were selected as an arbitrary reference point. In the adjacent marsh, 148 mussels were collected and their intertidal position was estimated with reference to the bridge.

Since small mussels are able to break byssus and redistribute themselves (Kuenzler, 1961), it was necessary to find the size beyond which no movement occurs. Mussels of 11 to 86 mm. were used to estimate rates of motility on horizontal and vertical surfaces. Individuals were placed in plastic pans containing sea water and horizontal distances traveled per unit time were measured. Rates of vertical movement were determined by placing single animals in sea water-filled glass cylinders with their anterior ends upward, and vertical distance traversed per unit time was measured.

Results

All mussels examined showed the same basic pattern of shell movements. When inundated, the valves are opened in small increments until a full-filtering position is attained. When exposed, the valves are immediately brought together to a nearly closed position and reopened equally rapidly to the air gape position. The air gape is about half as great as the full-filtering gape (Fig. 6). Superimposed on this basic pattern is another pattern of small, periodic movements by which the valves are opened or closed slightly. Both patterns are constant regardless of tidal period length. Further, air-gaping has been observed to be continuous for periods up to 121 hours.

During these experiments, a few mussels were tested for responses to crudely-applied photic and mechanical stimuli. Photic stimuli were applied with a microscope lamp and mechanical stimuli were light taps on the apparatus. The site of photosensitivity appears to be located in a row of darkly-pigmented, white-edged cells on the margin of the siphons. In air or water, mussels respond rapidly to either stimulus by closing. When the stimulus is removed, the valves are returned to their original position. A rapid reduction in light intensity elicits this response. Reapplication of this stimulus immediately after a response elicits a smaller, or no, second response. This attenuated response is usually known as habituation.

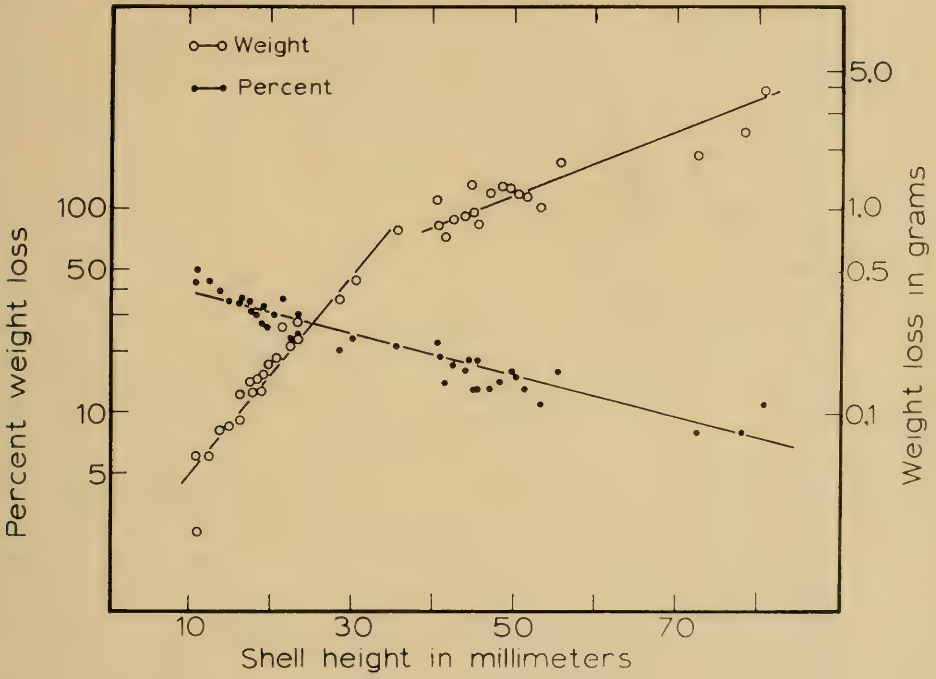

FIGURE 1. Semi-logarithmic plot of the per cent weight loss and actual weight loss as functions of shell height for 39 mussels after 49 hours of desiccation at 24.5° C. and 80% r.h. The weight losses are logarithmic and the shell height is arithmetic. The 4 smallest mussels in the plot are dead, the rest alive.

Charles (1966) noted that many bivalves have photosensitive mantles and Tauc (1966) reports that gastropods exhibit habituation to both photic and mechanical stimuli.

Five days was the median time to death for mussels in a nitrogen atmosphere. In air, the time increased to 9 days, and 13 days was observed for mussels in oxygen. Survival time is proportional to the volume of oxygen present. Kuenzler (1961) noted that these mussels can survive for 28 days out of water when protected from direct sunlight. Unquantitated observations indicate that aerial survival times may be on the order of months in cold, moist air.

The pattern of oxygen tension in mantle cavity fluid was uniform in all mussels tested. When exposed and gaping, the partial pressure of oxygen (pO_2) was low —10–18 mm. Hg. Upon inundation, the pO_2 had an average value of about 105 mm. Hg., fluctuating between 95 and 115 mm. Hg. After inundation, the pO_2 rises rapidly. Filtering rate appears to be governed by the size of the excurrent siphon aperture, and when open widest, the pO_2 was highest. When the aperture was constricted, the pO_2 fell. These fluctuations were not observed when mussels air-gaped. The pO_2 of external sea water varied between 120 and 145 mm. Hg.

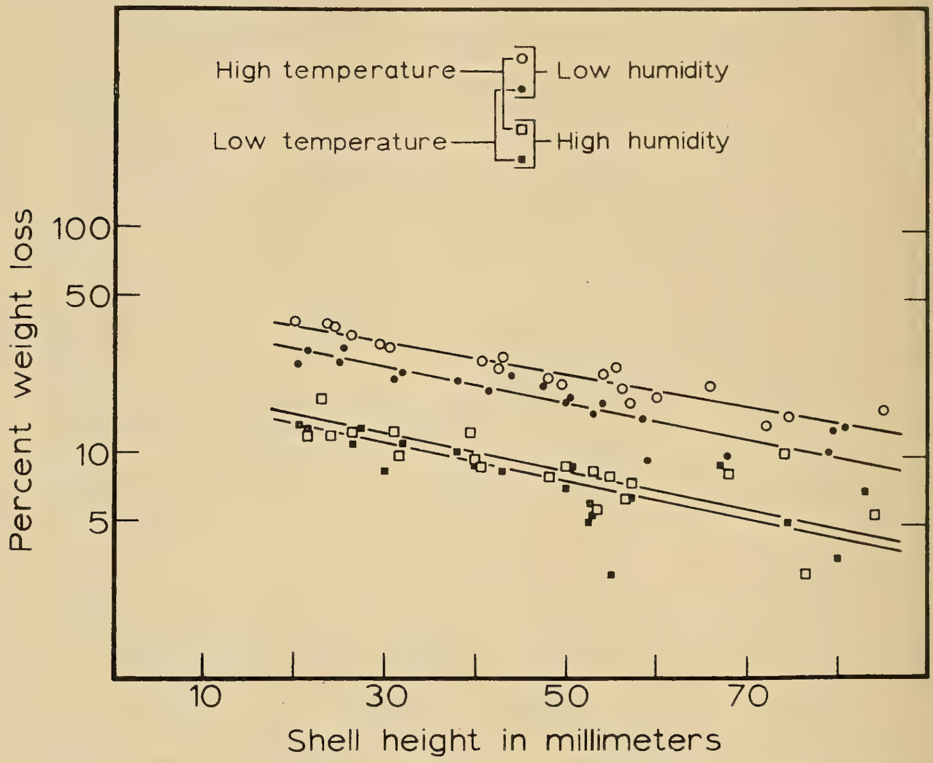

FIGURE 2. Semi-logarithmic plot of per cent weight loss as a function of shell height. Per cent weight loss is logarithmic. The temperatures are 24 and 29° C. and humidities are 75 and 100%. At 75%, the 5 smallest mussels are dead at 29° C. and the 1st, 2nd, and 4th smallest are dead at 24° C. All others are alive.

If per cent weight loss is expressed as a function of time, the rate of weight loss is greatest at time 0 and decreases logarithmically with time. The rate is highest in small mussels and decreases with increasing size. Figure 1 is a semi-logarithmic plot of both per cent weight loss and actual weight loss as functions of size for 39 mussels after 49 hours of desiccation in the first experiment. Per cent weight loss decreases with increasing size, but actual weight loss increases with size and this increase is greater in small than large mussels. An inflection exists in this relationship in the 35–40-mm. region. Kuenzler (1961) found an inflection in the same size range when comparing cube root of body weight to shell length. The 4 smallest mussels in the plot are dead, the rest alive.

Results of the second desiccation experiment are shown in Figure 2. It is a semi-logarithmic plot of per cent weight loss as a function of size. There are no significant differences between the slopes of these regressions. Table I contains the means of X and Y; the intercept, a, and the slope, b, of the estimating equation; the standard error of the estimate, SE; the coefficient of correlation, r; the coefficient of determination, r^2; and the F ratio for these regressions. The position of the slopes indicates that humidity difference had a greater effect on water loss than temperature difference. At low humidity, temperature difference had a significant effect on rate of desiccation, but not at high humidity. At 100% r.h., no mortalities occurred. But at 75%, the 5 smallest mussels died at 29° and the 1st, 2nd, and 4th smallest died at 24° C. It can be seen in these regressions that as the amount of desiccation increases, the variability decreases and correlation increases. This indicates that desiccation is the phenomenon being measured.

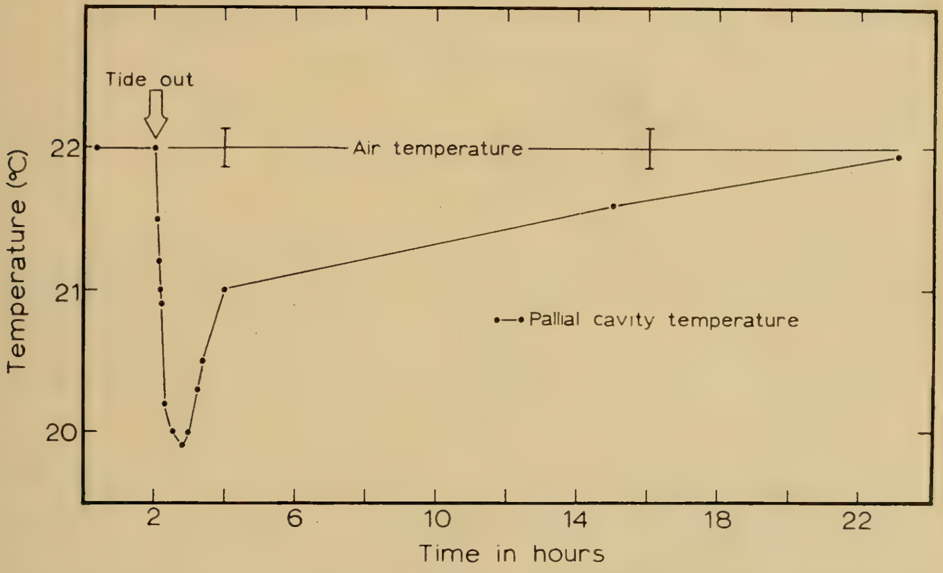

FIGURE 3. Pallial cavity temperature of an individual *Modiolus* during a simulated tide. External temperature is 22° C. and humidity is 60%. Vertical bars represent external temperature variation.

Several mussels died during desiccation experiments. Using all available data, per cent weight loss of both dead and live mussels, whose weight losses were of similar magnitudes, were plotted against per cent dead. Depending upon intervals chosen, the median death point from desiccation lies between 36 and 38%.

The pallial cavity temperature of a single mussel is shown for 23 hours after exposure in Figure 3. Similar patterns were observed for all tested, but this is the most complete plot for an individual. When the water is lowered, the internal temperature falls rapidly to a maximum observed depression of 2.1° C. after 30

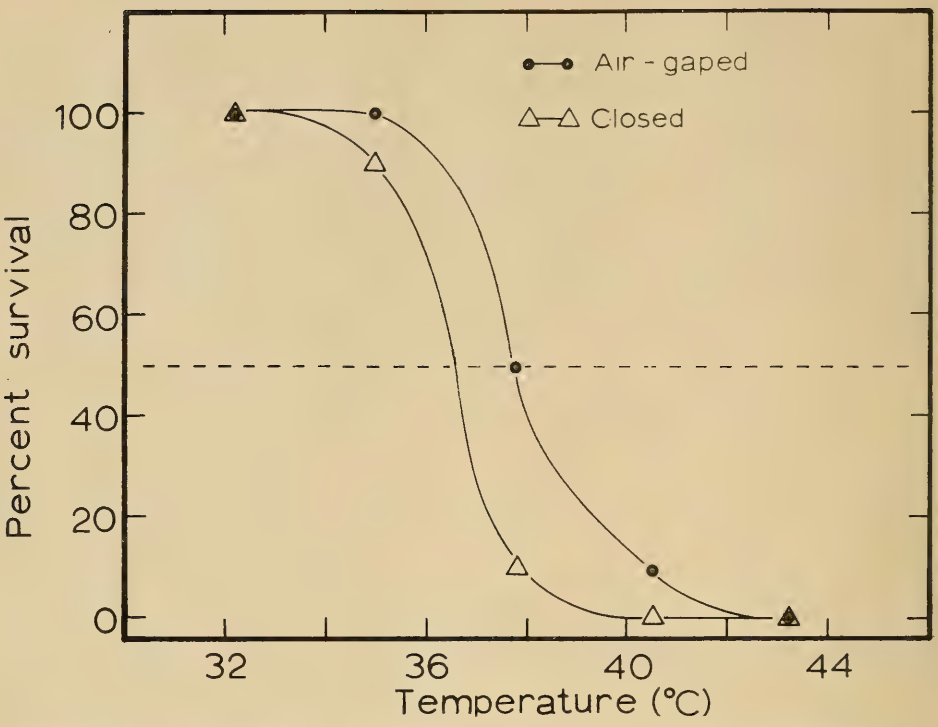

FIGURE 4. Per cent survival as a function of temperature. The stress was 10 hours and humidity was 80%. The dashed line intersects the curves at the LD_{50}.

minutes. The temperature then rises logarithmically, until after 23 hours, the mussel is essentially isothermal with the ambient air.

The effect of evaporative cooling on thermal survival is shown in Figure 4 which depicts per cent survival of each 10 mussel group as a function of stress

TABLE I

Regressions and constants of the log per cent weight loss as a function of shell height in millimeters after 68 hours of desiccation under 4 environmental conditions

$$\text{Log } Y = \text{Log } a + X \text{ Log } b$$

Humidity	75%	75%	100%	100%
Temperature	29°	24°	29°	24°
$\bar{X}$	46.22	46.31	47.29	48.07
$\bar{Y}$	1.380	1.258	0.942	0.876
a	1.734	1.590	1.291	1.221
b	−0.00771	−0.00719	−0.00737	−0.00717
SE	0.041	0.070	0.130	0.131
r	−0.957	−0.884	−0.742	−0.727
r^2	0.915	0.782	0.551	0.529
F	183	61	21	20

temperature. These data fit an expected tolerance-dosage curve very well—probit analysis gives a chi square of 0.0221 for for the air-gaped group and 0.0002 for the closed group. The LD_{50} by probit analysis is 37.8° C. for the air-gaped organisms and 36.4° C. for those closed. Graphical analysis gave values of 37.8 and 36.6° C., respectively. Probit analysis indicates, however, that the difference between these median death points is only slightly in excess of one probable error: this difference would be expected by chance alone nearly 50% of the time. Thus, there appears to be no significant difference produced by evaporative cooling on the high-temperature survival for a 10-hour stress.

When mussels were clamped and thermally stressed, the largest mussel in the test was always first to die and the smallest was always last, indicating that large mussels are more susceptible to heat stress than small ones. Thus, temperature mortality has an opposite effect on mussels of various size as desiccation mortality.

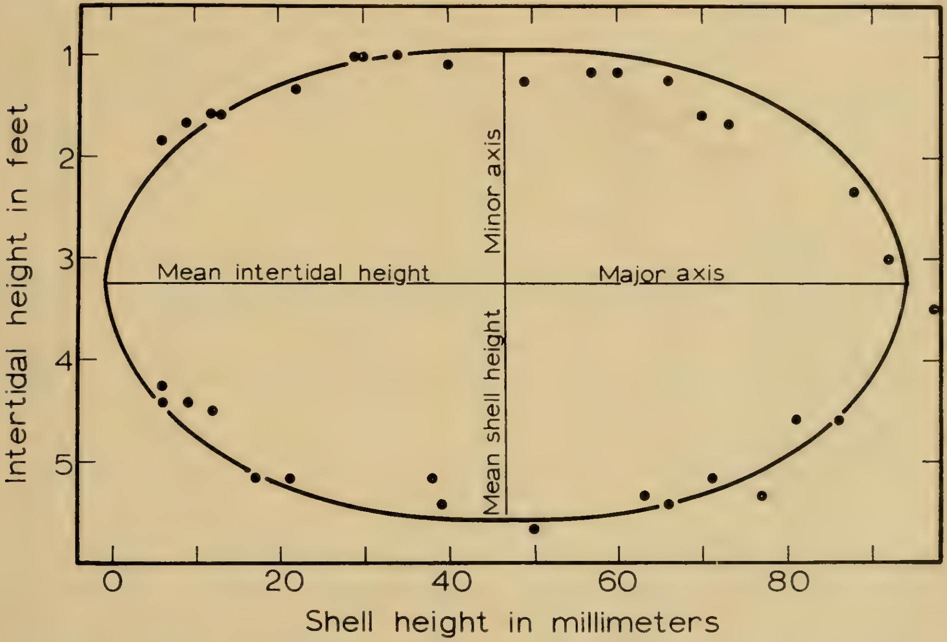

Figure 5. Intertidal height as a function of shell height for 33 mussels on the periphery of the distribution of Canary Creek Bridge.

Marsh mussels live within a 1-foot range at the mean high tide level. Bridge mussels have a vertical range of 4½ feet with an upper limit at the mean high tide level and a lower limit 10 inches below mean low tide level. The mean intertidal position of the bridge mussels is 3 feet below the marsh mussels. The bridge population is distributed normally with respect to intertidal height. If bridge mussels are grouped at 10-mm. intervals and the intertidal range of each group is plotted as a function of size, the intertidal range is seen to be greatest for medium-sized mussels of 30–60 mm. and is reduced for either larger or smaller groups.

This distribution pattern of bridge mussels falls within what appears to be an ellipse. To test this, the intertidal height of each bridge mussel was plotted as a function of size, and those mussels on the periphery were selected. The 33 points corresponding only to mussels on the periphery of the plot were fitted to the standard equation of the ellipse, $x^2/b^2 + y^2/a^2 = 1$, by the method of least squares. To perform this calculation, it was necessary to generate an origin at the center of the ellipse. The mean intertidal height of 39 inches was selected as y' and the mean shell height of 46.4 mm. was chosen as x'. The equation was then fitted with x' and y' as the origin using an IBM 1620 digital computer. The plot of the estimating equation and the 33 points from which it was calculated are shown in Figure 5. There is a respectable goodness of fit—a correlation coefficient of 0.936 and a coefficient of determination of 0.861. It is a significant regression with an F ratio of 192.

Motility rates were measured to find which areas in this distribution can be explained by mussel movement and which areas can only be explained by environmental selection. The highest rates are observed at 15–20 mm. (80 cm./hour) and are very low at sizes in excess of 45 mm. Rates are up to 100 times greater on horizontal than vertical surfaces.

Discussion

All responses investigated which are associated with air-gaping are shown as functions of an idealized tide cycle in Figure 6. Unless disturbed, the valves of ribbed mussels are always open and their tissues are always exposed. The generalized shell movement pattern shows the typical movement that immediately follows exposure. This movement is associated with the withdrawing of siphon margins before air-gaping.

The ability to respond to reductions in light intensity is probably a protective adaptation against predators when inundated or exposed. If a single mussel of a clump is disturbed, it closes and mechanical stimuli are transmitted *via* byssus to other individuals which respond in turn by closing. This may constitute a primitive type of communication affording mutual protection to all members of a clump.

Survival times in different gaseous environments suggest a utilization of atmospheric oxygen. However, a median lethal time of 5 days in nitrogen indicates tolerance of anaerobic conditions. Low oxygen is probably common when mussels get buried in marsh mud and this lethal time is not therefore surprisingly long. Kuenzler (1961) notes that this animal can endure anaerobiosis for extended periods and Hammen and Lum (1966) showed that some of the citric acid cycle enzymes in *Modiolus* are more efficient in an anaerobiotic than aerobiotic direction.

The pattern of pO_2 is depicted in Figure 6. The lowered pO_2 in air probably results from a reduction in the size of the gaseous exchange surface from the sea water-air interface to the mantle cavity water-air interface. It is reasonable to conclude that lowered rates of oxygen consumption in air observed by Kuenzler are due to lowered exchange surface.

These data indicate that desiccation is a physical phenomenon. Organisms which died during experiments had similar rates of desiccation to those which lived. In the second experiment the slopes were the same for all groups even though all mussels are alive in 2 regressions and some are dead in the other 2. Edney (1957) noted similar rates of water loss from live and dead *Ligia*.

For bodies such as spheres, per cent weight loss is proportional to the surface area/volume ratio if their membranes have identical physical properties. Making this assumption for mussels, the slope of the line, log per cent weight loss as a function of shell height, represents the change in surface area/volume ratio with increasing size. This assumption is strengthened by identical slopes under 4 different desiccation conditions. These slopes are displaced from one another by constants related to mean water loss.

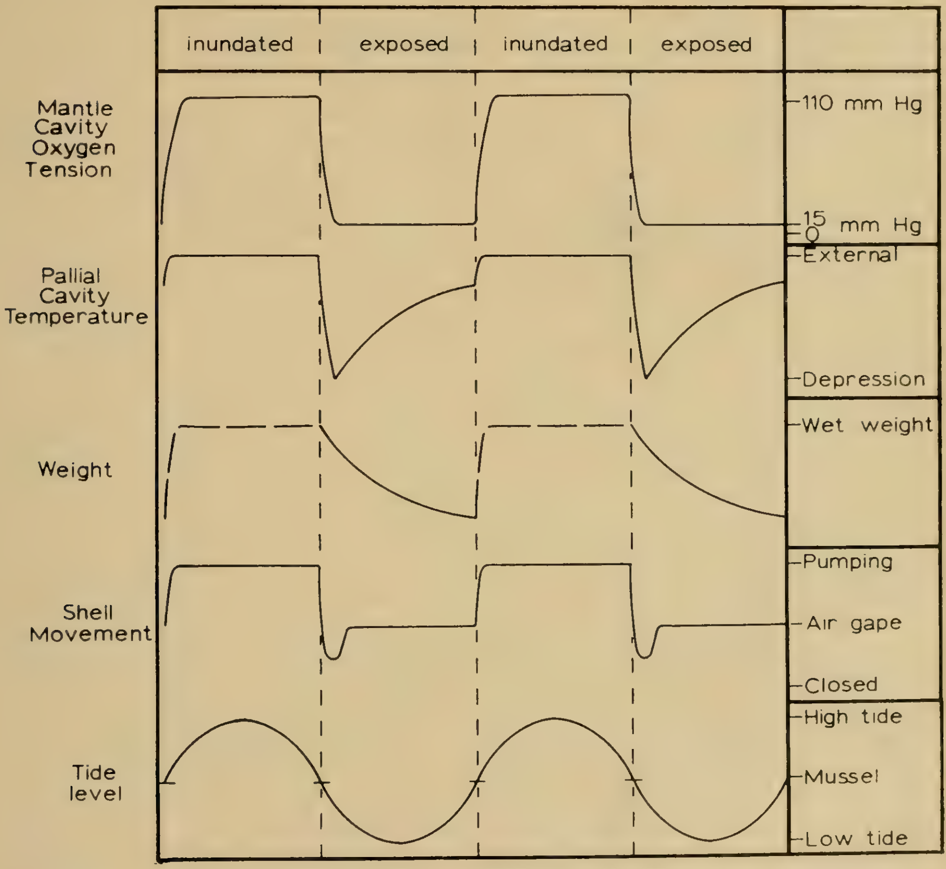
inundated
exposed
inundated
exposed
Mantle Cavity Oxygen Tension
Pallial Cavity Temperature
Weight
Shell Movement
Tide level
110 mm Hg
15 mm Hg
0
External
Depression
Wet weight
Pumping
Air gape
Closed
High tide
Mussel
Low tide

FIGURE 6. Responses associated with air-gaping by the ribbed mussel during idealized tides. A dashed line represents an unobserved response.

In organisms with low metabolic and high transpiration rates, most weight loss is probably water. Using a R.Q. of 0.85 (Kuenzler, 1961) and respiratory rates from Read (1962) and Kuenzler, metabolic weight losses were computed at 28 and 30° C. A 20-mm. mussel has maximum metabolic weight losses of less than 0.5% per day and a 100-mm. mussel less than 0.1%. Unlike water loss, these would be constant throughout the experiment and are insignificant compared to transpiration.

The LD_{50} of 36–38% for desiccation is reached more rapidly by small than large mussels because of the surface area/volume ratios. Frank (1965) showed in an intertidal snail population that larger snails tolerate drying better than smaller ones.

The greatest temperature depression occurs when evaporation is greatest (Fig. 6). As rate of water loss decreases, temperature is depressed less (*i.e.*, it rises). *Ligia* has a very similar water loss and cooling pattern. Maximum cooling is also reached after 30 minutes and its temperature then rises slowly (Edney, 1951). *Ligia* can be cooled more than 7° C. in dry air.

Evaporative cooling had no significant effect on high-temperature survival for a 10-hour stress; however, it is certain that greater cooling occurs in small mussels and at lower humidities. Transpiration may affect survival only during short-term stress since maximum cooling occurs during the first hour of exposure. Edney (1957) considered this cooling to be beneficial to *Ligia*.

The LD_{50} for temperature lies between 36.4 and 37.8° C. for a 10-hour stress. Vernberg, Schlieper and Schneider (1963) showed that gills of this bivalve function up to 44° C. for 100 minutes, and Read (1963) noted that the aspartic-glutamic transaminase system could withstand 56° C. for 12 minutes without significant reduction in activity. Read (1964) noted that several molluscs have enzymes whose thermal inactivation temperatures are up to 20° C. higher than environmental temperatures. However, Orr (1955) showed that as time of stress increased, the death temperature decreases in a wide variety of marine invertebrates. These 3 data generate a curve very similar to those of Orr.

The ellipse is an interesting feature of bridge mussel distribution. The clumping behavior in the marsh precludes this pattern. The major axis of the ellipse is generated by growth, and the minor axis by the outward movements of young mussels and inward environmental selection. Two factors interact to produce the curve of the upper limit and these data suggest that both are physical: desiccation is deleterious to small mussels and heat affects the large ones. The lower limit is formed in a similar fashion with 2 factors interacting. Although there are no data in this study which suggest what these factors are, some conjecture is in order. Connell (1961) demonstrated that the barnacle *Chthamalus* is limited on its lower level by competition with *Balanus* with these facts: *Chthamalus* has greater resistance to heat and/or desiccation; time of submergence is not responsible for the elimination; and *Balanus* has greater growth and metabolic rates. Parallels to these statements can be drawn between *Modiolus* and *Mytilus edulis* which lives in the lower intertidal zone of the bridge. Thus, there is an inferred case for competition as one of the factors; however, 2 factors affect the lower limit. Competition would affect larger mussels with their lower weight-specific metabolism and sexual maturity which places them under an energetic load. Small *Modiolus* have very thin shells and predation probably sets their lower limit. Many studies indicate that predators preferentially seek out and prey upon small bivalves (Blake, 1960; MacPhail, Lord and Dickie, 1955; McDermott, 1960; and Menzel and Nichy, 1958). This study is a substantiation of the conclusions of Frank (1965) and Connell (1961) that physical factors set the upper limit of intertidal populations. Their conclusion that biotic factors set lower limits of these populations may also be the case here.

The ellipse is analogous to the temperature tolerance polygon for fish (Fry, 1947). The polygon is a geometric figure which describes the zone within which a fish can live indefinitely with regard to 2 factors: acclimation temperature and stress temperature. The ellipse is a geometric form within which a natural population lives. It is generated by a minimum of 4 factors and is the resultant of all environmental effects.

The distribution of the same species in the marsh is different. Marsh mussels live much higher, but their range is reduced. Marsh mud has physical properties which are important to a mussel living in it. Primarily the mud is moist and the humidity in this microhabitat approaches 100%, reducing desiccation. Thus, small mussels survive 2 to 3 feet higher intertidally in the marsh than on the bridge. Secondarily, marsh mud is cooler than the air above: 5 cm. below the surface, temperatures can be up to 6.7° C. cooler on a hot summer day and differences of 5° C. are common. Intimate contact with this cool substrate reduces thermal stress and allows large mussels to avoid the high air temperatures at low tide. Observations (Lent, 1965, unpublished) and the data of McDougall (1943) indicate that such differences in distribution between marsh and exposed mussels also exist in the Beaufort, North Carolina, region.

If all responses of an organism are adaptive, air-gaping must have a selectively advantageous function. Cooling probably plays a minor role, especially in marsh mussels. Most likely, it is a mechanism for gaseous exchange. Since *Modiolus* spends more time exposed than inundated, access to oxygen would allow more efficient respiration. Baird and Drinnan (1957) hypothesize that *Mytilus* is limited to the low intertidal zone by its metabolic energy requirements when exposed and closed. If correct, air-gaping is the factor allowing *Modiolus* to penetrate the high intertidal habitat.

Modiolus is well-adapted for high intertidal dwelling: it utilizes oxygen in air and water and is tolerant of wide variations in temperature and salinity. Nevertheless, more terrestrial penetration appears unlikely because of the bivalve body plan: it is a filter feeder, it reproduces by planktonic gametes and larvae, and is sessile except when inundated. Thus, it is linked to the watery habitat, and adaptations to intertidal life have been made on the physiological level.

This work constitutes part of a dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences. I thank my advisor, Dr. Richard A. Nystrom, for his advice and ideas in this study; Dr. Henry B. Tingey for assistance with statistical analyses, and Dr. Harald B. Rehder for confirmation of the identification of the ribbed mussels.

SUMMARY

1. Normally, the intertidal mussel, *Modiolus demissus*, does not close its valves when exposed to air by low tides: it air-gapes. Desiccation resulting from water loss is a physical phenomenon and a surface-to-volume relationship causes small

mussels to reach the median lethal weight loss of 36–38% more rapidly than large mussels.

2. Evaporative cooling is demonstrable, but no significant difference in high tempertaure survival was observed between gaped and non-gaped mussels. The LD_{50} for a 10-hour heat stress lies between 36.4 and 37.8° C. and large mussels are more labile than small ones to thermal stress.

3. Median survival times of mussels in gaseous environments are proportional to the volume of oxygen present. Observations of lowered rates of respiration in air are explained by lowered oxygen tensions in mantle cavity fluid in air-gaping mussels.

4. Mussels living in moist marsh mud occupy a higher intertidal position than those living on bridge pilings which are more exposed to the aerial environment. When the intertidal position of bridge mussels is examined as a function of size, the population lies within an ellipse. The upper surface is generated by physical factors: desiccation and thermal stress. Predation and competition are proposed as biotic factors responsible for the lower surface.

5. Air-gaping is probably an adaptation for aerial respiration with desiccation as an unavoidable side effect.

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THE EFFECTS OF LOW TEMPERATURE, AND CORTISOL, ON TESTICULAR REGRESSION IN THE HYPOPHYSECTOMIZED CYPRINODONT FISH, FUNDULUS HETEROCLITUS

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The role of environmental temperature in the regulation of the reproductive cycle of teleostean fishes has been subjected to repeated investigation. The earlier literature was reviewed by Atz (in Pickford and Atz, 1957), and updated in a bibliographic review (Atz and Pickford, 1964); to these may be added a recent article by Ahsan (1966a) on the lake chub, *Couesius plumbeus.*

Warm temperature plays a primary role in stimulating the maturation of the gonads in *Fundulus heteroclitus* (Burger, 1939; Matthews, 1939b). Conversely, low temperature retards and high temperature accelerates the rate of post-spawning regression in this species (Burger, 1940), and in others (Egami, 1956; Ahsan, 1966a). Hypophysectomy, which simulates the effects of post-spawning regression (Matthews, 1939a; Burger, 1941), has been little investigated in relation to temperature. Preliminary observations on hypophysectomized *F. heteroclitus,* maintained at 10° C. and 20° C., indicated that the degree of sexual regression, reflected in the gonadosomatic index, was less severe at the lower temperature (Pickford, in Pickford and Atz, 1957). However, this finding was not accompanied by cytological studies such as those recently reported for hypophysectomized *F. heteroclitus* maintained at 20° C. (Lofts, Pickford and Atz, 1966).

In the present investigation, we have studied the effects of low temperature on the testes of long-term hypophysectomized male *F. heteroclitus,* and the effects of a subsequent return to warm temperature. Since it has been suggested by Scott (1964) that stress, mediated through the pituitary-adrenal axis, is responsible for post-spawning regression in the ovaries of *Phoxinus phoxinus,* we also investigated the effects of cortisol administered to the partially regressed fish at low temperature.

MATERIAL AND METHODS

Male *F. heteroclitus,* captured in the vicinity of New Haven, Connecticut, in May, 1964, were maintained for 4 months at 15° C., in sea water, with 8 hours per day illumination and daily feeding, as described previously (Pickford, 1953,

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1959). At the time of capture, they were undergoing the initial stages of sexual maturation. Full maturity was attained during the following weeks in captivity. The fish were hypophysectomized between July 22 and 24, 1964. At this time, in striking contrast to a parallel group maintained at 20° C., the nuptial colors were still fully developed and the majority of fish had flowing sperm.

The temperature was gradually lowered between August 29 and September 1, and the fish were maintained at *ca.* 10° C. until February 14, 1965. At this time, approximately 29 weeks after hypophysectomy, traces of nuptial coloration were still evident. In contrast to a parallel group of hypophysectomized fish maintained at 20° C., the group at 10° C. had not passed into complete sexual regression. The latter fish were then divided into 4 experimental groups:

Group I. Initial controls. Autopsied February 15, 1965.

Group II. The temperature was gradually raised, between February 14 and 16, to 20° C. The treatment was evidently highly stressful, and 4 of the 9 fish assigned to this group died during the first 2 days. The survivors were maintained at 20° C. until the termination of the experiment.

Group III. Terminal controls. Maintained at 20° C.

Group IV. Maintained at 10° C. Untreated until April 6, and then given intraperitoneal injections of cortisol, to simulate natural stress, on alternate days for 2 weeks prior to autopsy.

The dose of cortisol was calculated to be at a stressful but not pharmacological level (0.025 μg. per gram weight of fish, suspended in 0.01 ml. of 0.6% NaCl). If one assumes that the material injected into the body cavity is uniformly distributed in an extracellular space of the order of 15%, this dose would be approximately equivalent to the known circulating level of cortisol found by Phillips (1959) in what were undoubtedly highly stressed males of this species (15.2 μg./100 ml. plasma). We have since found that this dose is highly stressful in eliciting a leucocytic response in hypophysectomized male *F. heteroclitus* at 20° C. (Pickford, Slicher and Pang, unpublished).

All fish were weighed and measured at the time of hypophysectomy, and again both at the start and at the termination of the definitive experiment. No fish that showed any significant increase in length (1 mm. or more) on February 14 was assigned to the experiment; some showed a small length decrement, but this is usual after hypophysectomy. Nuptial colors were rated according to the arbitrary scale employed in previous investigations (Lofts, Pickford and Atz, 1966): "slight trace" or "trace" indicates a barely detectable tinge of yellow on the pelvic fins; "±" indicates yellow on both pelvic and anal fins; higher ratings, when the yellow spreads forwards over the belly, were not encountered in the present investigation.

Groups II, III, and IV were autopsied on April 29, 1965, one day after the last cortisol injection in Group IV. Each fish was lightly anesthetized with MS 222, weighed and measured. A blood sample was taken from the cut end of the tail. The testes were weighed on a Mettler balance, and bisected; one-half was fixed in sea-water Bouin's fluid, the other in Baker's formol saline. Liver weight was also recorded. Testis and liver weights are expressed as percentage of body weight (gonadosomatic and hepatosomatic indices, respectively).

RESULTS

A. *General*

Fish maintained at 10° C., in the absence of the pituitary, became severely anemic; cortisol had no beneficial action, but the return to 20° C. restored the circulating levels of erythrocytes, leucocytes and thrombocytes to "normal" levels for hypophysectomized *F. heteroclitus* at this temperature. These results have been reported in a separate communication (Slicher and Pickford, 1968).

The relative size of the liver was essentially the same in all 4 groups; average values for the hepatosomatic index ± standard error are as follows: Group I, 7.66 ± 0.54; Group II, 6.11 ± 0.54; Group III, 6.41 ± 0.09; Group IV, 7.22 ± 0.76. Large liver size, reflected in hepatosomatic indices of this order of magnitude, is characteristic of hypophysectomized *F. heteroclitus* and of intact individuals in sexual regression. The present findings show that all fish, in all groups, were in healthy condition.

The data on nuptial coloration and the gonadosomatic index are summarized in Table I. Each fish is listed individually, with its identification number, to facilitate comparison with the cytological condition of the testes. It is apparent that sexual regression proceeded very slowly at 10° C. The mean gonadosomatic index seems to have decreased slightly between $5\frac{1}{2}$ and 8 months, but the difference is not statistically significant. Return to 20° C. (Group III) resulted in an almost total disappearance of nuptial coloration and a significant decrease in the relative size of the testes. The gonadosomatic index, 0.257, is appropriate to that previously reported for hypophysectomized male *F. heteroclitus* at 20° C. Cortisol had no significant effect on the relative size of the testes.

B. *Cytological Studies*

The histological and histochemical methods employed were the same as those used in previous investigations (Lofts, Pickford and Atz, 1966). The degree of spermatogenetic activity was estimated by counting the total number of germinal cysts of each spermatogenetic stage found in five randomly selected cross-sections of testis lobules. The mean for each spermatogenetic stage was then estimated for each experimental group and plotted as a histogram (Fig. 1). The different stages are:

Ia. A single primary spermatogonium.
Ib. A cyst of primary spermatogonia.
II. A cyst of secondary spermatogonia.
III. A cyst of primary spermatocytes.
IV. A cyst of secondary spermatocytes.
V. A cyst of spermatids.
VI. A cyst of spermatozoa.

Initial Control Group (Group I). All the specimens in this group had a testis in which there were numerous cysts of all spermatogenetic stages (Fig. 2). There were numerous cysts of spermatozoa, but no spermatozoa in the efferent ducts. There were clusters of pycnotic nuclei showing that some of the cysts were becoming necrotic, but these were not present in large numbers. Mitotic figures

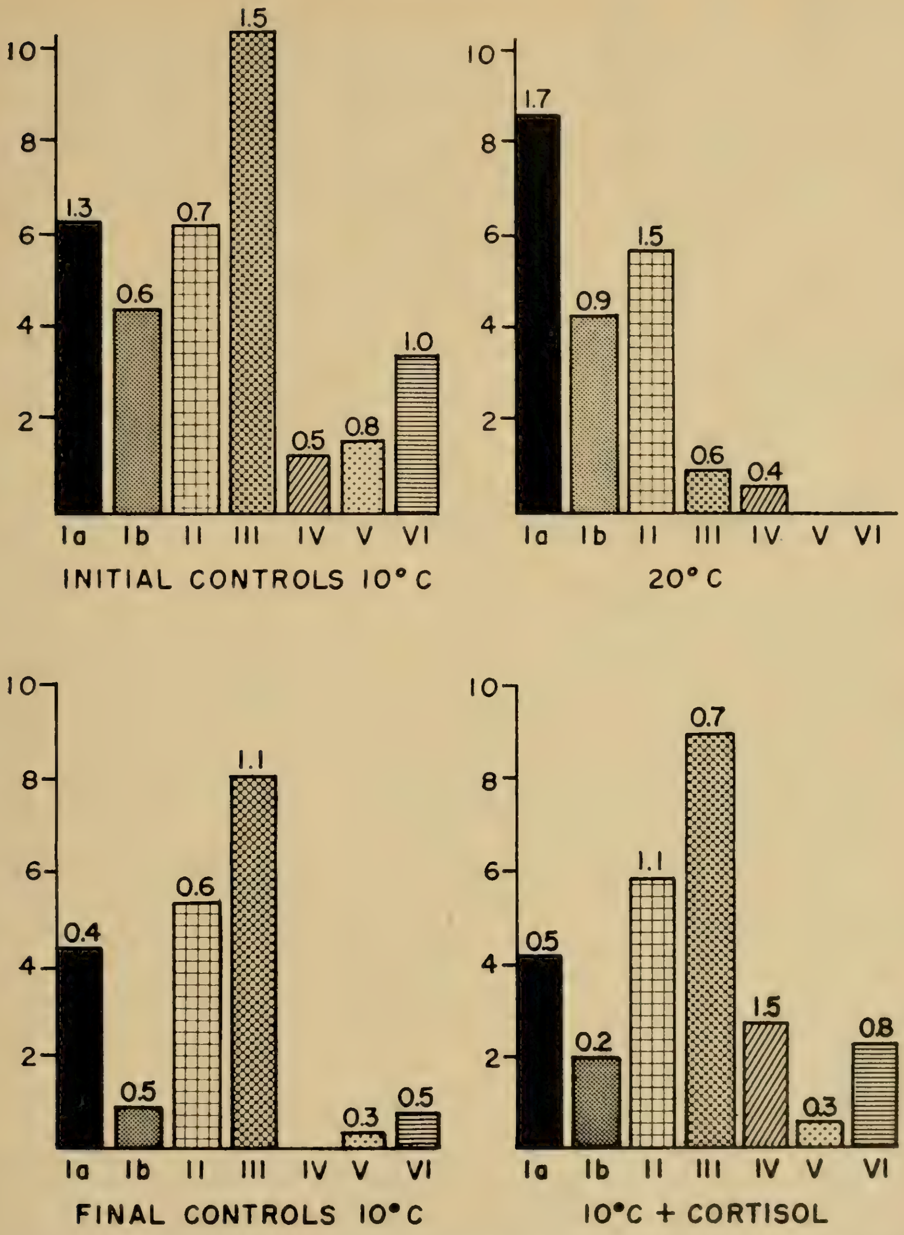


FIGURE 1. Spermatogenetic condition of hypophysectomized male *Fundulus heteroclitus* at 10° C. and 20° C., and after treatment with cortisol. Each histogram represents the mean number of each germinal stage in 5 cross-sectioned lobules. See text for description of the different spermatogenetic stages (Ia–VI). Figure on top of each bar is Standard Error.

TABLE I

Nuptial colors and gonadosomatic indices of hypophysectomized male Fundulus heteroclitus, *under experimental conditions*

Group	Fish	Nuptial color*	Gonadosomatic index†
I. 10° C. for 5½ mo.	154	Trace	0.812
	179	Trace	0.931
	181	Negative	1.401
	182	±	0.682
	184	Negative	0.632
	193	Trace	0.612
	Average ± SE		0.845 ± 0.122
II. 10° C. for 8 mo.	156	Slight trace	0.707
	173	Slight trace	0.656
	177	±	0.473
	186	±	0.722
	189	Trace	0.434
	190	Slight trace	0.345
	Average ± SE		0.561 ± 0.063
III. 20° C. for last 10 wks.	158	Negative	0.241
	159	Negative	0.151
	167	Slight trace	0.253
	171	Negative	0.294
	178	Negative	0.347
	Average ± SE		0.257 ± 0.032
IV. 10° for 8 mo., cortisol last 2 wks.	155	Negative	0.327
	163	±	0.832
	165	Slight trace	0.568
	166	Slight trace	1.144
	170	Slight trace	0.629
	172	Slight trace	0.528
	178	±	0.960
	Average ± SE		0.712 ± 0.106

*Arbitrary rating; see text.
† Weight of testis × 100/Body weight.

in the primary spermatogonial region were rare. The interstitial tissue contained some rounded nuclei and there was slight development of the efferent ducts.

20° C. Group (Group II). Elevation of the temperature had accelerated the rate of regression in all specimens except No. 178 and the germinal zone contained only cysts of spermatogonia and isolated primary spermatocytes. In specimens Nos. 167 and 171 this spermatogonial zone was about 3–4 layers deep (Fig. 3), but in Nos. 158 and 159 the regression was more marked and spermatogonial cysts formed only a thin peripheral layer to the gonad (Fig. 4). In specimen No. 178 cysts of all spermatogenetic stages were still present, although the post-spermatogonial stages were numerically very few. No stage V or VI cysts occurred in the 5 sectioned lobules used for counting the germinal stages. These observations show close agreement with the gonadosomatic indices (Table I).

In all specimens, mitotic figures in the primary spermatogonia were much more common than in Group I. This suggests an increase in the rate of primary spermatogonial division, and this is supported by the histogram (Fig. 1) which shows an elevation of primary spermatogonial numbers with a concomitant reduction of other stages.

Pycnotic nuclei were very common in this group and all gonads contained large quantities of necrotic material. The interstitial nuclei were shrunken, irregular and dense; only in specimen No. 167 were a few large, rounded nuclei seen. The efferent duct system of all specimens was regressed.

Terminal 10° C. Group (Group III). The members of this group had still not reached a fully regressed state. Division of the primary spermatogonia, which was not uncommon at 20° C., continued to be retarded, and only in specimen No. 177 was a single mitosis observed in these cells. In specimen No. 186 there were numerous cysts in all stages, and this specimen could be taken for a member of the original control group (Group I). The gonadosomatic index was also higher in this specimen.

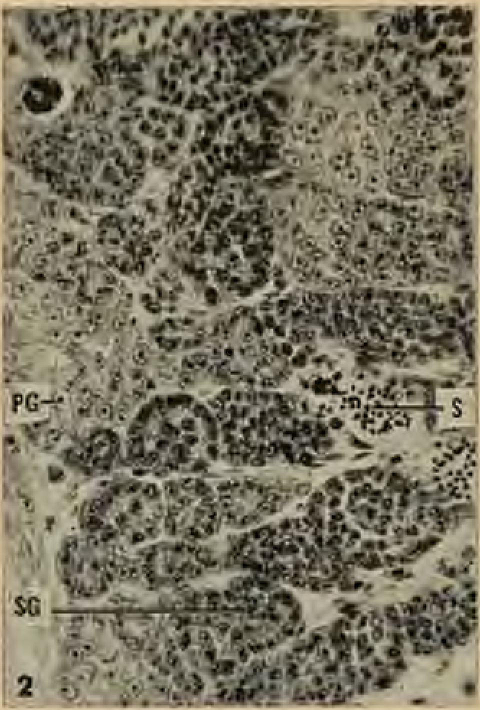
In specimens No. 173 and 176, all spermatogenetic stages were also represented but the total number of cysts was markedly fewer than in the initial controls (Fig. 5). As the original "reservoir" of spermatogonial cysts present in these gonads at the time of hypophysectomy slowly matured without, or with only very slow, replacement by primary spermatogonial divisions, a gradual reduction in the total number of cysts occurred throughout the duration of the experiment. The April specimens, therefore, contained fewer cysts than the February fish. This also explains the fact that advanced stages such as primary spermatocytes and even a few sperm cysts were often found in the peripheral layer of primary spermatogonia. Generally, such stages are separated from the testis periphery by a zone of spermatogonial cysts (Fig. 2).

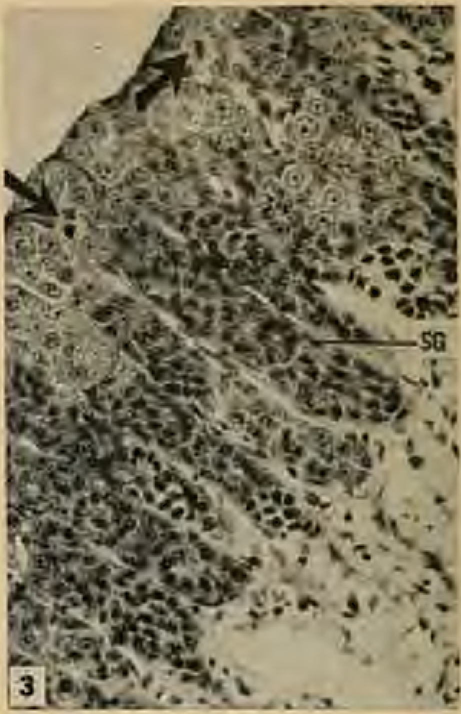
In specimens No. 189, 177 and 190, all stages beyond primary spermatocytes had degenerated. The testes of all specimens contained numerous degenerating cysts with pycnotic nuclei. Accumulations of necrotic material were also present.

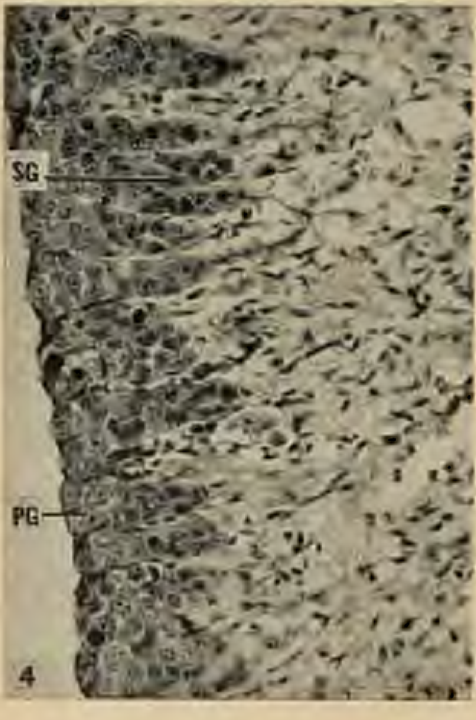
There were numerous regressed nuclei in the interstitial tissue but there were also rounded nuclei with a conspicuous nucleolus still present, suggesting some level of secretory activity. A slight development of the duct system also points to a measure of hormonal activity.

Cortisol and 10° C. Group (Group IV). The members of this group appeared slightly less regressed than the low-temperature fish that had not been injected (Group III). Specimens No. 175, 163, and 166 contained all spermatogenetic stages and were almost like the initial controls. They differed from the latter in the presence of a few spermatocyte cysts at the periphery of the gonad. The remaining four specimens of this group had a germinal zone consisting mainly of cysts of secondary spermatogonia and primary spermatocytes, with only a relatively few isolated cysts of more advanced stages. Some necrotic material was present in all specimens in this group.

Spermatogonial mitoses were rare. In specimen No. 155, the interstitial cells







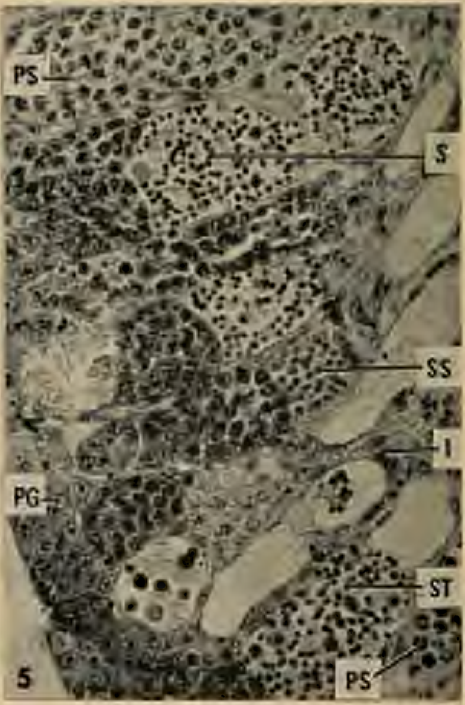

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PLATE I

appeared to be fully regressed, as did the efferent duct system, but in all others the interstitial tissue had not reached a fully regressed state and gave the appearance of being secretory. The efferent ducts also showed some development, but no more than in the terminal 10° C. group.

Cyst degeneration and removal of necrotic material. When the testis regresses, germinal cysts degenerate. This degeneration appears to affect the primary and secondary spermatocytes first, probably during meiotic division. The cyst is then seen to contain a cluster of pycnotic nuclei (Figs. 4 and 6). As more and more cysts degenerate, they accumulate into larger masses (probably by phagocytic action) in the testis stroma (Figs. 6 and 7). These masses become a compact ball of necrotic cells surrounded by a thin fibrous wall (Fig. 7). The ball then moves to the surface of the testis, passes between the lobules and becomes extruded into the body cavity (Figs. 8–11).

Such bodies are present at all times of year in the testes of wild *F. heteroclitus* (previous observations), but are most common during post-spawning regression. Matthews (1938) recorded clusters of cells with shrunken, pycnotic nuclei that became prominent at this period. In the present experiment, the process was most obvious in the 20° C. group, which is to be expected since it was in this group that regression had been accelerated.

It is these clusters of necrotic cells that Matthews suggested were probably the interstitial tissue. This suggestion has been refuted by Lofts, Pickford and Atz (1966), and the above described process also disproves it.

DISCUSSION

While it is generally agreed that the testes of hypophysectomized teleostean fishes pass into a state of regression, there are comparatively few studies of the cytological changes. The older literature includes the work of Matthews (1939a) and Burger (1941) on *Fundulus heteroclitus,* and Vivien (1941) on *Gobius paganellus.* More recent investigations are those of Barr (1963) on *Pleuronectes platessa,* Roy (1964) on *Ophicephalus punctatus,* Ahsan (1966b) on *Couesius*

PLATE I

Testicular tissues of hypophysectomized *Fundulus heteroclitus* stained with iron hematoxylin and orange G. Bouin fixation; × 375. PG = primary spermatogonia; SG = secondary spermatogonia; PS = primary spermatocytes; SS = secondary spermatocytes; ST = spermatid; S = spermatozoa; I = interstitial tissue.

FIGURE 2. Testis of fish maintained at 10° C. for 5½ months. Germinal cysts of all spermatogenetic stages, including spermatozoa, still numerous. The spermatogonial zone is wide (*cf.* Fig. 5).

FIGURE 3. Testis of fish maintained at 10° C. for 5½ months and then 20° C. for 10 weeks. Testicular regression has been accelerated and post-spermatogonial stages have now disappeared. Primary spermatogonia in mitotic division (arrows) are more common. Interstitial cell nuclei are small and condensed.

FIGURE 4. Testis of fish treated as in Figure 3. Regression is more advanced in this specimen and the residual zone of spermatogonia is greatly reduced.

FIGURE 5. Testis of fish maintained at 10° C. for 8 months. Germinal cysts in all stages are still present but reduced in numbers. There are fewer spermatogonial cysts than after 5½ months (*cf.* Fig. 2), and advanced spermatogenetic stages are commonly present in the peripheral area. Many interstitial nuclei are still rounded and conspicuous.

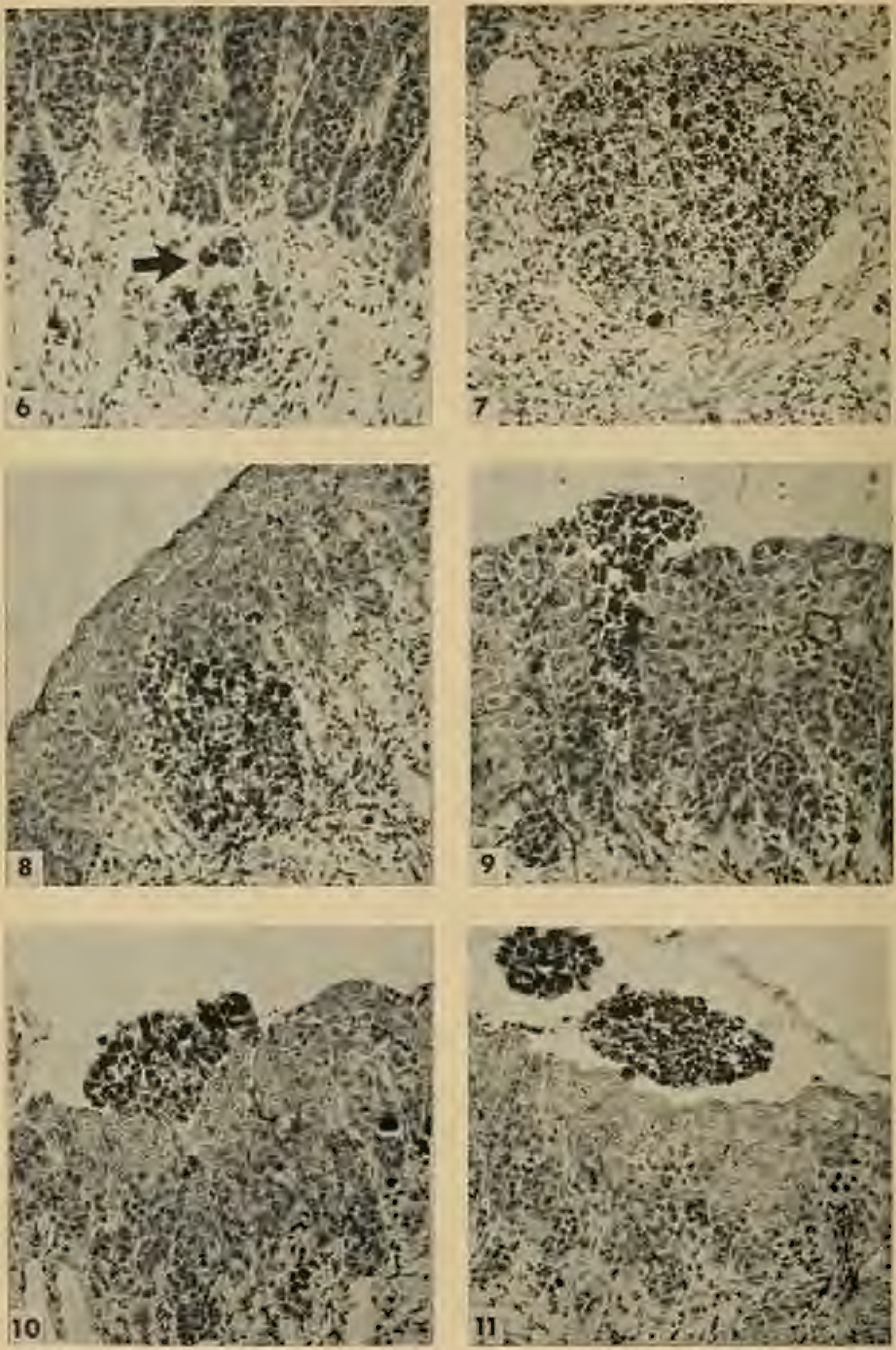

PLATE II

plumbeus, and Sundararaj and Nayyar (1967) on *Heteropneustes fossilis*. To these we may add the information contained in our earlier paper on the effects of methyl testosterone on the regressed testes of *Fundulus heteroclitus* (Lofts, Pickford and Atz, 1966).

It is generally conceded that considerable time is required for the gonads of hypophysectomized fish to pass into complete regression. In *Gobius paganellus*, for example, Vivien observed slow regression during a period of 5 to 6 months, and at least 2 months were required in *Fundulus heteroclitus* (Burger, 1941). Some investigators have reported the temperature at which their experiments were made, but, in general, this aspect of the problem has been neglected. Burger states that his fish were kept at 11–19° C., mostly below 15° C.; Ahsan captured his fish at different times of year, when the environmental temperatures were widely different, but his studies on the effects of hypophysectomy were made at 18–21° C., and the period of acclimation was rather short (30–40 days). Barr maintained his fish in running sea water which, according to an earlier paper, varied from 3.3° C. in winter to 17.8° C. in summer. The experiments of Sundararaj and Nayyar (1967) were made at constant temperature (25° C.).

All investigators agree that hypophysectomy is followed by a cessation of maturation that affects the abundance of spermatocytes. Ahsan believed that the critical stage is the one that immediately precedes the transformation of secondary spermatogonia into primary spermatocytes. Barr suggested that the first meiotic division is the one that is inhibited. Spermatogonial mitoses, on a reduced scale, have been observed by a number of investigators, and the consensus is that spermatogonial multiplication is retarded but not abolished. On the other hand, it is clear that when maturation is well advanced at the time of hypophysectomy, spermiogenesis and sperm release continue. With time, partly as a result of this process, and partly through necrotic changes such as those described by Ahsan and by ourselves, the testes become depleted of spermatocytes, spermatids, and spermatozoa. These stages may persist for long periods of time, however; in three of the 10 hypophysectomized *Pleuronectes platessa* that he investigated, Barr observed spermatozoa in the regressed testes 130, 175, and 222 days after hypophysectomy. The operations were made in January, February, and March, presumably at low temperature, and this may have retarded the speed of regression. However, Sundararaj and Nayyar (1967), working on the Indian catfish *Heteropneustes* at warm temperature (25° C.), observed residual sperm that persisted even 337 days after hypophysectomy.

Our results are in essential agreement with the picture outlined above. At 10° C. the rate of testicular regression is retarded, but not halted. The rate of spermatogonial multiplication is also retarded. Interstitial cell activity continues

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PLATE II

Testicular tissue of hypophysectomized *Fundulus heteroclitus*, maintained at 10° C. for 5½ months and then at 20° C. for 10 weeks, showing the mechanism of extrusion of necrotic cysts; × 250.

FIGURE 6. Degenerating germinal cysts (arrow) are collected by phagocytic action into compact masses.

FIGURE 7. The necrotic material is condensed into a tight ball in the testicular stroma.

FIGURES 8–11. The necrotic material is extruded between the lobules into the body cavity.

at a low level, and, presumably in correlation with this, traces of nuptial coloration are retained. There is a gradual reduction in the number of germinal cysts as spermatogonial cysts already present at the start of the experiment slowly progress into more advanced stages and degenerate. There is little or no replacement of spermatogonial cysts since the mitotic rate of the primary spermatogonia is retarded.

The condition described above is profoundly affected by a return to warm temperature. The rate of post-spermatogonial regression is accelerated, so that more degenerating cysts are seen and these are extruded from the testis by the process described. In striking contrast, however, warm temperature increases the rate of spermatogonial mitosis and the number of primary spermatogonia increases. Interstitial cell activity ceases, and the last traces of nuptial coloration disappear.

These experiments suggest a direct effect of temperature on the gonad. Since an elevation of temperature accelerates spermatogonial activity, it is possible that in *Fundulus heteroclitus* the rising temperature in early spring initiates a greater responsiveness of the testes to pituitary stimulation. This aspect of the problem requires further investigation. In *Couesius plumbeus* the situation appears to be somewhat different. Ahsan (1966a) found that high temperature accelerates the proliferation of spermatogonia in intact fish, and hastens or terminates spermiation, but low temperatures are essential for what appears to be the pituitary-dependent phase, the formation of primary spermatocytes. In another paper Ahsan (1966b) failed to find any consistent difference in the response of the testes of hypophysectomized *Couesius* to gonadotropins at low and high temperature, although the gonadosomatic index was higher at low temperature.

In summary, the results of the present investigation point up three aspects of the role of temperature in the regulation of the reproductive processes of poikilotherms: (1) A direct effect on the gonad, (2) an indirect effect on the gonad, regulating its ability to respond to pituitary stimulation, and (3) effects on pituitary synthesis and release of gonadotropins.

Cortisol, at the dose and under the conditions employed, had no significant effect on the efferent duct system or on the rate of spermatogonial multiplication. The rate of testicular regression was possibly less severe than in the uninjected controls; regression was not accelerated, as had been anticipated. Previous investigations of Rasquin and Hafter (1951) and Rasquin and E. H. Atz (1952) demonstrated that high doses of cortisone acetate, or of ACTH, stimulated the sperm duct epithelium of intact *Astyanax mexicanus,* while spermatogenesis was decreased. Our experiments with a physiological dose of the naturally occurring adrenal corticoid have failed to confirm this observation on hypophysectomized *Fundulus heteroclitus.* However, in view of the recent work of Sundararaj and Goswami (1966) on ovulation in the catfish, *Heteropneustes fossilis,* a possible role of adrenal corticoids in teleostean reproduction cannot be excluded.

SUMMARY

1. Low temperature retarded the rate of sexual regression in hypophysectomized male *Fundulus heteroclitus.*

2. Transfer to warm temperature accelerated the rate of post-spermatogonial regression, and the partially regressed interstitial tissue became inactive.

3. In contrast, the return to warm temperature stimulated the rate of spermatogonial mitosis. It is suggested that temperature has a direct action on spermatogonial multiplication.

4. Cortisol, at a physiological dose, had no significant effect on the condition of the testes.

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THE PERMEABILITY OF THE EPIPHRAGM OF TERRESTRIAL SNAILS TO WATER VAPOR

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Terrestrial snails owe their survival in dry environmental conditions to the protection afforded by the shell during periods of inactivity. Although some water passes through the shell, the rate of evaporative loss is minimal (Machin, 1967). Water loss by way of the aperture is greatly reduced by some kind of regulatory mechanism (Machin, 1966), but is also influenced by the nature of the shell aperture and associated structures. One of the most important of these is the epiphragm which is usually secreted across the open aperture once the snail becomes inactive, leaving an air-filled space between it and the mantle (Fischer, 1853; Allman, 1896; Taylor, 1894–1907). The term *epiphragm* in this context is used in its broadest sense. It refers to the heavily calcified plate or "pomum" (Woodward, 1913), "poma" (Hora and Rao, 1927) or "winterdeckel" (Flössner, 1915) secreted for example by *Helix pomatia* in winter, also to the much thinner membranous structure or "mucous veil" (Howes and Wells, 1934) or "hybernaculum" (Hora, 1928) produced by *Helix pomatia* in summer and by many other species including *Helix aspersa* and *Otala lactea.*

The epiphragm consists of an organic matrix of dried mucus in which are embedded variable amounts of calcareous granules. The organic component in heavily calcified epiphragm is 9.7% by weight in *Achatina fulica* (Mead, 1961) and 6.8% by weight in *Helix pomatia* (Taylor, 1894–1907). In some of the thinner epiphragma, inorganic granules are almost entirely absent. Histochemical studies (Campion, 1961) of glandular tissue in the mantle collar suggest that the epiphragm, in *Helix aspersa,* is composed of mucopolysaccharide associated with protein. Mucoprotein is absent while the inorganic component seems to be predominantly calcium carbonate.

There is evidence (Machin, 1962) that dried epiphragmal mucus differs somewhat from the mucus which normally coats the snail. Freshly extruded mucus readily swells in distilled water and eventually disperses. However, once the epiphragm has dried after secretion, it remains a completely coherent membrane, having hydrofuge surface properties when in contact with water.

This paper describes a method of determining epiphragm permeability to water vapor, and attempts to relate results to evaporative water loss from the aperture of the intact inactive snail.

METHODS AND RESULTS

Epiphragm permeability

Epiphragma were removed from inactive specimens of *Helix aspersa, Helix pomatia* and *Otala lactea* (all terrestrial helicids) with the aid of a sharp scalpel.

The apparatus for measuring permeability to water vapor (Fig. 1) consisted essentially of two glass chambers separated by the epiphragm, through which air was passed at constant rate. Each air stream was dried in a column of silica gel and then equilibrated to a given vapor pressure by bubbling it through an appropriate saturated salt solution, selected from the data given by Spencer (1926) and Winston and Bates (1960). The entire apparatus is immersed in a water bath maintained at 25° C. ± 0.01° C.

At the beginning of each determination the isolated epiphragm was clamped between the ground rims of the chambers. An air-tight seal was obtained with a small amount of silicone grease. The rigid, plate-like winter epiphragm of *Helix pomatia* was sealed with wax in a metal mounting to facilitate clamping. Each

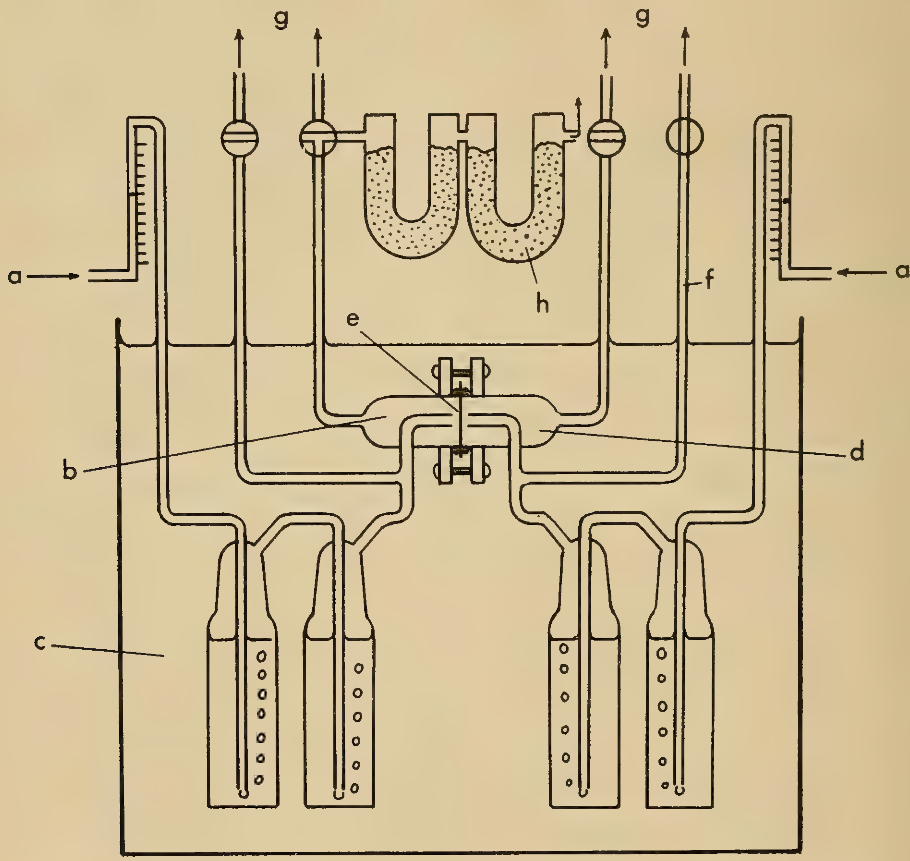

FIGURE 1. Diagram of apparatus used for measurement of epiphragm permeability coefficients. Stopcocks which were used to control the flow of air through various parts of the apparatus are shown at the top of the drawing: (a) air in through flow meters; (b) "dry side" of epiphragm, *e.g.* using saturated $CaCl_2$ solution, vapor pressure = 7.2 mm. Hg; (c) water bath at 25° C.; (d) "wet side" of epiphragm, *e.g.* using saturated $ZnSO_4$ solution, vapor pressure = 20.4 mm. Hg; (e) epiphragm; (f) epiphragm by-pass; (g) to dew-point hygrometer; (h) "U" tubes filled with silica gel.

epiphragm was pressure-tested for possible leakage before and after the experiment. Air flow either side of the membrane was adjusted to 1.5 liters/min. with the aid of graduated-tube flowmeters. Preliminary experiments established that this rate of flow was sufficiently rapid to abolish the vapor gradients in air close to the epiphragm. Measurements were made with several vapor pressure gradients across the membrane. The vapor pressure was measured by re-routing each air stream through a recording Cambridge Systems dew-point hygrometer before it reached the membrane. Dew-point measurement of the outflowing air stream of the "dry" side of the epiphragm also provided a useful criterion for the establishment of equilibrium. At the beginning of the experiment the dew-point temperature steadily rose to a plateau in about one hour for thin epiphragma and in about four hours for the thick epiphragm of *Helix pomatia.* Once this equilibrium had been established, the dry outflowing air stream was routed through a preweighed double "U" tube filled with freshly dried 6 to 16 mesh silica gel. The amount of water left unabsorbed was considered negligible since the dew-point of the emergent air stream was less than −40° C. The amount of water which had passed through the membrane in a given time, usually several hours, was calculated from the difference in the weight of water extracted from "dry" air stream before and after it had passed over the membrane. The thickness of each epiphragm was determined with a micrometer.

Data from some 26 determinations were used to calculate the permeability coefficient k, which may be defined as the weight of water in mg. diffusing in one hour across an area of 1 $cm.^2$ when there is a fall in vapor pressure across the membrane of 1 mm. Hg in 1 mm. The results showed that the value of k was not constant, but varied with different vapor pressure gradients. It was found that the epiphragma were more permeable when the average of the vapor pressures on either side of the membrane was high. Assuming a linear vapor pressure gradient this mean value is equivalent to vapor pressure halfway through the membrane, and represents its average water content. The relationship between mean vapor pressure and k is shown graphically in Figure 2.

The data were analyzed with a view to establishing which of a number of possible mathematical relationships best fitted the data. However this was impossible due to the large variability in corresponding values of k.

The effect of the epiphragm on water loss from the intact snail

It was stated above that the measurements of epiphragm permeability coefficient must be made in the absence of vapor pressure gradients in the air either side of the membrane. However vapor pressure gradients, particularly in the air space between epiphragm and mantle surface, may have an important effect on evaporation from the intact snail. Accordingly, rates of diffusion of water vapor through the epiphragma of *Helix aspersa* and *Helix pomatia* were determined in still air in the following manner. Isolated epiphragma were sealed with epoxy resin across the ends of short lengths of glass tube, 1.2 cm. in diameter. The other ends of the tubes were closed with a rubber bung. Different amounts of distilled water were introduced into the tubes through the rubber with a hypodermic syringe. The distance between the water surface and the epiphragma varied between 3.0 and 0.5 cm. The epiphragma were punctured with a minute hole to equalize the

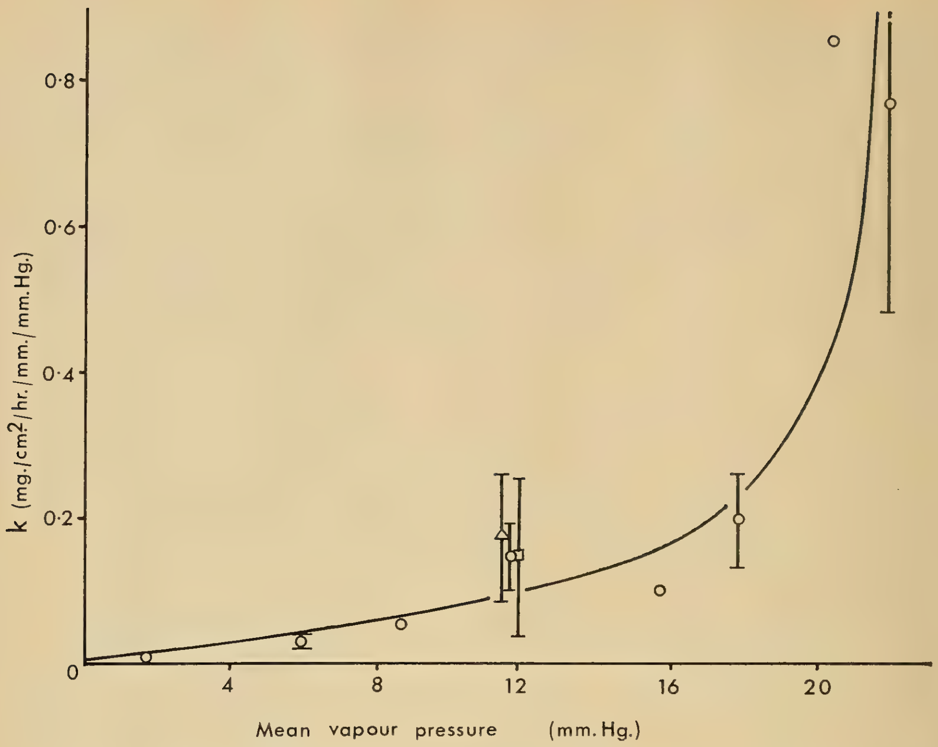

FIGURE 2. Graph showing change in epiphragm permeability, k, with different mean vapor pressures across the membrane. Epiphragma removed from different snail species are indicated thus: *Helix aspersa* (○) *Helix pomatia* (□) and *Otala lactea* (△). Vertical bars represent ± S.E.

pressure on either side of the membrane. Evaporative loss at an ambient temperature of 25° C. was determined gravimetrically. The results obtained are given in Table I and are compared with evaporation data from identical water-filled glass tubes with open ends. The data in Table I show that evaporative loss is reduced by the epiphragma; however, loss is not simply related to membrane thickness, it varies also with the length of the air space beneath the epiphragma. Loss through the thinner epiphragm of *Helix aspersa* is influenced by the dimensions of the space much more than in the case of the thicker epiphragm of *Helix pomatia.*

Results obtained from the experimental models together with evaporation data from intact snails, taken from Machin (1967), were also used to calculate the permeability coefficient for each epiphragm in still air. These values will be termed "apparent permeability coefficients" since vapor pressure gradients in the air either side of the epiphragma were not taken into account. The vapor pressure drop across the epiphragm, used in the calculation, was simply obtained by subtracting the vapor pressure of ambient air from the vapor pressure at the evaporating surface. Values for surface vapor pressure in the experiment of models

TABLE I

Rates of evaporation in still air from glass tubes, open or sealed with isolated epiphragma. Rates of evaporation are expressed in mg./cm.²/hr. and have been corrected for slight differences in temperature and humidity at the time of each measurement. Rates have been calculated for a vapor pressure gradient of 13.57 mm. (34% RH) in air temperatures of 25° C.

Distance between evap. surface and end of tube. (cm.)	Evaporation Rates				
	Open tube (no epiphragm)	Epiphragm of *Helix aspersa* (thickness 0.038 mm.)	% reduction in evap. rate	Epiphragm of *Helix pomatia* (thickness 0.65 mm.)	% reduction in evap. rate
3.0	3.8	3.3	13	0.99	70
2.0	5.0	3.9	22	1.14	78
1.0	8.9	7.7	13	1.50	83
0.5	17.3	13.9	20	1.49	91

was obtained from tables (Handbook of Chemistry and Physics, The Chemical Rubber Company) giving the saturated vapor pressure of distilled water at the temperature at which the measurements were made. Corrections for evaporative cooling, which would be small, were not made. Mantle surface vapor pressures in intact snails were taken from Machin (1966). It has already been argued

TABLE II

Percentage differences between apparent and actual permeability coefficients for different types of epiphragma under different experimental conditions. Conditions which are considered "membrane-limited" are indicated thus:.*

Type of epiphragm and state of animal	Distance between epiphragm and evap. surface (cm.)	Epiphragm thickness (mm.)	Mean v.p. across epiphragm (mm. Hg)	Actual k from Fig. 2	Apparent k	% Difference
Helix aspersa (in experimental model)	3.0	0.038	12.7	0.110	0.0092	91
	2.0	0.038	12.7	0.110	0.011	90
	1.0	0.038	12.0	0.100	0.022	78
	0.5	0.038	11.0	0.085	0.051	28*
Helix pomatia (in experimental model)	3.0	0.650	12.7	0.110	0.075	32*
	0.5	0.650	11.0	0.085	0.073	14*
Helix aspersa (regulating)	1.0 (approx.)	0.046	3.5	0.023	0.022	4*
Otala lactea (regulating)	1.0 (approx.)	0.110	3.5	0.023	0.019	17*
Sphincterochila boissieri (regulating)	1.0 (approx.)	0.299	3.5	0.023	0.020	13*

(Machin, 1967) that the use of these data for related species is reasonable. Ambient vapor pressures were derived in all cases from dew-point temperature measurements made at the time of each experiment.

In Table II "apparent permeability coefficients" are compared with actual permeability coefficients, determined in the absence of vapor pressure gradients in air. It follows if the apparent coefficient is no different from the value obtained in moving air, vapor pressure gradients in still air are negligible. Under these conditions the epiphragm provides the greatest resistance to diffusion, *i.e.*, the system is "membrane-limited" as defined by Beament (1961). However if the two permeability coefficients differ greatly, vapor pressure gradients in air are important and the system may be described as "vapor-limited." Since there can be no hard and fast rule distinguishing between membrane- and vapor-limited conditions, the point at which gradients in air can no longer be considered negligible must be arbitrarily decided. In the present study it was thought for practical purposes, discrepancies of up to 32% in the apparent permeability coefficient could be considered negligible (see Table II).

Discussion

The results of measurements made with the epiphragm confirm previous frequently made assumptions (see Taylor, 1894–1907, *inter alia*) that the epiphragm reduces water loss from the shell aperture. However, the extent of this reduction is probably not as great or as important as earlier studies suggest. Hogben and Kirk (1944) found that *Helix pomatia,* with an intact epiphragm, has the same body temperature as the surrounding atmosphere. It is not clear whether they thought this indicated a completely unpermeable epiphragm or not. In the light of more recent studies, this interpretation is almost certainly incorrect. Machin (1966) found that inactive snails are able to reduce evaporative loss from the skin by a regulatory mechanism which is still poorly understood. The data of Hogben and Kirk, including slight increases observed in evaporative cooling when the epiphragm is removed, are entirely consistent with a theory of "regulation" and also with the results of the present study.

The determined coefficient of epiphragmal permeability is seven times greater than that of the shell under the same vapor pressure conditions (Machin, 1966). It is three orders of magnitude more permeable than hydrocarbon waxes under similar vapor pressure gradients, one order of magnitude more permeable than various rubber compounds. The epiphragm is similar in permeability to "waterproof cellulose film" (see Taylor *et al.,* 1936). Concentration-dependent permeability has been found to be a characteristic of many different types of organic membranes including proteins (see Beament, 1961; Machin, 1962; Christensen, 1965). Taylor *et al.* (1936) obtained almost exactly the same relationship between diffusion coefficient and vapor pressure conditions with a membrane of "soft vulcanized rubber." This change in diffusion coefficient is usually explained by an alteration in the molecular structure of substances which sorb or swell as the concentration of the diffusant increases.

A limited number of permeability measurements were made with epiphragma in opposite directions. No asymmetry of the type described by Beament (1961) was evident, indicating that the structure and properties of the epiphragm are rather uniform throughout its thickness. The uniformity of permeability coefficients of epiphragma from three different species, which varied considerably in thickness and the amount of calcium carbonate present, suggests that diffusion is governed by the organic component of the membrane. The permeability of a given epiphragm simply varies inversely with thickness.

Complete analysis of the factors involved in evaporative loss from the aperture of an inactive snail in a way which incorporates all the proper permeability or diffusion coefficients is extremely difficult. The difficulty is due to the fact that a system in which water vapor must sequentially diffuse through a series of solid and gaseous phases is inherently complex. Results have shown that water loss from the snail can only under special circumstances be considered as a simple problem of diffusion through the epiphragm. In conditions where diffusion along vapor pressure gradients in air must also be taken into account, it is difficult if not impossible, without directly measuring these gradients, to formulate appropriate equations from fundamental principles. The crux of the problem is due to the fact that the vapor pressure gradient beneath the epiphragm depends on epiphragm permeability which, in turn, depends on the vapor gradients. It may be of some value to note that standard procedures for the measurement of permeabilities of other membranes under similarly complex conditions by the "control dish method" (Turl and Kennedy, 1965) now avoid direct determination of permeability coefficient altogether.

It has been possible in the present study to establish under what conditions the measurement of epiphragm permeability provides a direct indication of evaporative loss from the aperture of the intact snail. A possible interpretation of the results obtained is presented in diagrammatic form in Figure 3. "Membrane-limited" conditions are found in "regulating" snails, where vapor pressure gradients in air must be small (see Fig. 3A). It has been shown (Machin, 1966) that the vapor pressure at the surface of inactive snails is normally very low. Thus "membrane-limited" conditions may be attributed to the fact that the vapor pressure gradient

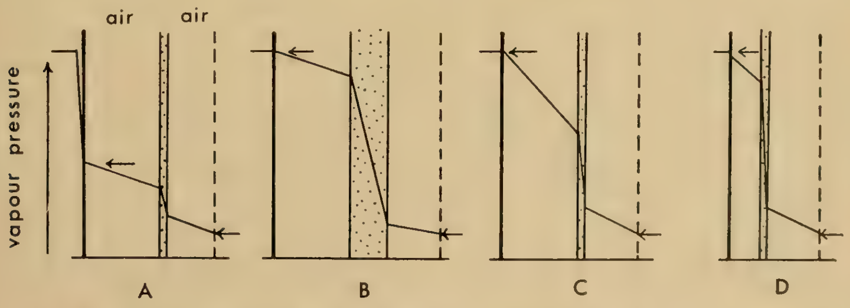

FIGURE 3. Diagram illustrating assumed vapor pressure gradients in different evaporating systems. The evaporating surface is to the left of each diagram. The epiphragm, whose thickness has been exaggerated, is stippled; the approximate limit of the vapor boundary layer in air is indicated by the interrupted line. Vapor pressures used to calculate apparent epiphragm permeability coefficients are shown by the arrows. Condition A: any regulating snail, thick or thin epiphragm. B: *Helix pomatia* epiphragm either in model or in stimulated animal. C: stimulated *Helix aspersa*. D: experimental model, distance between surface and epiphragm small. Conditions A, B and D are considered "membrane-limited."

between blood and the surface occurs within the animal itself. When surface vapor pressure is high, membrane-limited conditions may also be found, as long as the epiphragm permeability is low enough to abolish the substantial gradient across the intervening air space. In practice these conditions are satisfied in the presence of the thick epiphragma of *Helix pomatia* (see Fig. 3B). With thinner and more permeable epiphragma in the model, membrane-limited conditions are not obtained (Fig. 3C), except, perhaps, when the length of the air space is small enough (0.5 cm.) for the drop in vapor pressure along its length to be negligible (see Fig. 3D).

Financial support from the National Research Council of Canada (Operating Grant A1717) is gratefully acknowledged.

SUMMARY

1. A method for the measurement of epiphragma permeability is described.

2. Permeability coefficients varied greatly, depending on the water content of the epiphragm.

3. Permeability coefficients of epiphragma from different species of snail were similar at a given water content.

4. Experiments using models with thick and thin epiphragma indicated that evaporative loss in still air was not always related to epiphragm permeability in a simple manner.

5. Conditions under which epiphragm permeability provides a reasonable indication of evaporative loss in the intact snail are indicated.

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RESPONSES OF THE FALSE LIMPET, SIPHONARIA PECTINATA LINNAEUS (GASTROPODA, PULMONATA) TO OSMOTIC STRESS[1]

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The presence of an external shell or shells in molluscs lends to this group a unique potential for a rather specialized behavioral response during osmotic stress. This response consists of temporary exclusion of the environment by clamping or closing of the valves; it has been suggested to occur in several different species of molluscs as a mechanical method of osmoregulation. The principle involved here is quite similar to that shown by the eggs of marine and fresh-water fish. In this case, the eggs are protected against osmotic stress by the fact that they are impermeable to both water and salts. Thus a barrier against equilibration during periods of concentration differences is erected.

Among the molluscs, Krogh (1939) demonstrated that the bivalves *Mytilus, Crassostrea,* and *Modiolus* respond for varying lengths of time to transfer from normal to dilute sea water by closing the valves. With the valves closed, these marine pelecypods could survive and maintain the hemolymph undiluted for several days. However, if the valves are forced open, the animal swells rapidly and little volume regulation is observed. Among these bivalves, *Mytilus* seems to show some degree of volume regulation, since Krogh (1939) found that the hemolymph and tissue chloride concentrations had become reduced in dilute sea water. Further evidence that *Mytilus* is able to tolerate dilute sea water was furnished by Milne (1940), who found that, at low tide, the salinity inside the mantle cavity of *Mytilus* was 24‰, while the salinity of the environment had fallen to 7‰. Freeman and Rigler (1957) demonstrated that the estuarine lamellibranch *Scrobicularia plana* tolerates wide salinity variations by closure of the valves, seriously decreasing the rate of equilibration of the body fluids with the external milieu after environmental concentration changes.

Among the gastropods, Mayes (1962) found a gradual increase in weight in all the species of British *Littorina,* an operculate snail, upon transfer of the animals to 25% sea water. In this medium, all animals remained retracted until death; Mayes therefore concluded that the survival times of each species in this medium were correlated with the goodness of fit of the operculum. Avens and Sleigh (1965) have verified these findings using *Littorina saxatilis.* These workers concluded that hyperosmoticity of the body fluids of this animal in dilute solutions is a result of avoidance of deleterious conditions by retraction, rather than active hyperosmotic regulation, since active animals were always isosmotic with the medium. Thus, Avens and Sleigh concluded that the retraction must be of con-

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siderable importance in the survival of the animal under temporary adverse conditions.

Among the prosobranch limpets, Milne (1940) suggested that *Patella vulgata* may have the ability to exclude the environment during periods of osmotic stress. Arnold (1957, 1959) suggested that *Patella* does show such responses during desiccation, and proposed that the cephalic tentacles and/or mantle fringe contain receptors for the perception of salinity. Segal (1956) and Segal and Dehnel (1962) gave evidence that extravisceral water may serve an osmoregulatory function in *Acmaea limulata*.

The animal selected for the present study, *Siphonaria pectinata*, has not been previously investigated as to its capacities for volume and/or ionic regulation, with the exception of a study done by Harry and Bucher (unpublished). These workers demonstrated that the chloride concentration of the hemolymph closely paralleled that of the external milieu at all experimental concentrations. Weight fluctuations were found to be less than those predicted for a perfect osmometer.

This study was undertaken to examine the clamping response in the false limpet, that is, the active contraction of the foot and shell muscles to reduce exposure of the soft parts of the animal to the environment. Experimental design was constructed in an effort to establish a normal salinity range for the animal, and experiments were conducted on both attached and unattached animals, in hopes of producing some measure of the efficiency of the clamping response in reducing volume fluctuations in osmotic gradients. Although the false limpet has many features distinct from the prosobranch, or true limpet, it is hoped that this study will produce evidence in support of the observations of earlier workers, that limpets can indeed respond to osmotic stress by using the substratum as an "auxiliary valve," in a fashion similar to that demonstrated to be operative in bivalve molluscs.

Materials and Methods

Collection and maintenance of animals

Animals were obtained along the South Jetty, Galveston, Texas, which is located at the northeastern tip of Galveston Island. The jetty lies due west of the mouth of the Houston Ship Channel. All animals were collected at low tide from the south, or Gulf, side of the jetty from a point approximately one-fourth mile from the beach.

The collection technique consisted of the introduction of a dull knife or scalpel under the shell and foot, and exertion of a gentle prying force. Care was taken to prevent damage to the shell fringes or underlying soft parts. A small amount of water from the collection site was also collected, for salinity measurements.

The animals were placed in an ice chest with a small amount of ice for transport to the laboratory. Upon arrival, they were individually placed, foot down, in recovery aquaria (henceforth referred to as "tanks"), each of which contained 15 gallons of "Instant Ocean" (Aquarium Systems, Inc., Cleveland, Ohio) artificial sea water. Animals were distributed among the recovery tanks so that there were approximately 50–75 animals per tank. All tanks, both recovery (control) and experimental, were aerated and an external charcoal-glass wool filter was attached to each tank. The salinity of the control tanks was maintained at 24‰. All artificial sea water solutions were prepared by diluting a concentrated stock solution of the artificial sea water to the desired salinity. Salinities were measured with hydrometers and corrections determined with conversion tables supplied by the Coast and Geodetic Survey, U. S. Department of Commerce. All experiments were conducted in an air-conditioned laboratory (22–26° C.).

Before an experiment, animals were acclimated in the recovery tanks for at least 48 hours. Mortality during the first 24 hours was 19%. This was attributed to either (1) injury inflicted during collection or (2) shock inflicted either during transfer or after exposure to the artificial sea water. After this 48-hour period, animals were maintained with little difficulty in the recovery tanks.

Collection of hemolymph samples

For removal of body fluids, the animal was first blotted dry with absorbent paper, and the sole of the foot was carefully lacerated with a scalpel near the lateral margin, thus puncturing the foot sinuses and ventral hemocoel. This method was only fairly successful, however, and an average of 40% of all animals tested yielded either no hemolymph at all or else a fluid of a brownish color. Subsequent determinations on the hemolymph utilized only those animals which yielded a clear bluish fluid. The difficulty of obtaining body fluid samples was most marked in animals acclimated to extreme hypoosmotic conditions. In these cases, only one animal in five yielded a measurable amount of hemolymph. Conversely, in hyperosmotic solutions, the animals yielded hemolymph quite readily.

Hemolymph samples were collected with 25-microliter micropipettes. Since fluid volumes from individual animals varied between 5 μl. and 300 μl., depending on the size of the animal and salinity of the water from which the animal was taken, pooled hemolymph samples from a minimum of three animals were used for all determinations. These pooled samples were stored in glass vials at −20° C., and the samples were taken from these vials for the determination of Δt_f and Na^+ concentration. A sample of the water from which each of the experimental groups was taken was also collected in vials and stored at −20° C. Each time a quantity of the hemolymph was used in a determination, an equal amount of the sea water in which that group of animals was maintained was also measured.

Measurement of Δt_f

The capillary technique used for the measurement of the freezing point depression (Δt_f) was a modification of that described by Gross (1954).

Na^+ determinations

The determinations for Na^+ concentrations were made in the following manner. Five-microliter samples of hemolymph and an equal amount of the appropriate sea water were taken from the pooled storage vials and diluted 1:1000 in 7% ethanol in 5-ml. polyethylene containers. A series of standard solutions of sodium chloride in 7% ethanol were also prepared: 0.1 m*M*, 0.25 m*M*, 0.5 m*M*, 0.75 m*M*, 1.0 m*M*, and a blank of 7% ethanol. The standards were determined using a

5-second response time on a Zeiss Spectrophotometer M4 QII equipped with a flame photomoter attachment. Absorbances were plotted *versus* concentration and a standard curve was obtained. The experimental samples containing hemolymph or appropriate sea water were then measured and their Na^+ concentrations determined from the standard curve.

WEIGHT DETERMINATIONS

For the determination of wet weights, the animals were blotted dry with absorbent paper and weighed on a torsion balance.

RESULTS

EXPERIMENTS INVOLVING TRANSFER OF ANIMALS INTO DIFFERENT SALINITIES

This group of experiments was designed in an effort to determine the animal's osmotic behavior when transferred from normal sea water to sea water of a "stress salinity," that is, a salinity outside the range normally encountered by the animal.

TABLE I

Per cent survival and per cent attachment. Animal transfer experiments.

S‰.	24 hours		48 hours		72 hours		96 hours	
	%S*	%A**	%S	%A	%S	%A	%S	%A
5	10	0	0	—	—	—	—	—
10	25	0	0	—	—	—	—	—
20	100	90	90	90	90	90	90	90
30	100	100	100	100	100	100	100	100
40	90	85	85	85	85	85	85	85
50	60	40	40	40	40	40	40	40
60	0	0	—	—	—	—	—	—
24	100	100	100	100	95	95	95	95

* Per cent survival.
** Per cent attachment.

Groups of 20 animals were removed from the control tanks (24‰) and placed into plastic containers, containing sea water at different salinities, ranging from 5‰ to 60‰ at 10‰ intervals. Thus each experiment involved eight containers holding salinities 5, 10, 20, 30, 40, 50, 60‰, and one container for the control salinity 24‰. After 24 hours exposure to the experimental salinity, five animals were removed, body fluids withdrawn and pooled, and the Δt_f values determined. The per cent of the animals attached to the bottom of the container after 24 hours was recorded, as was per cent survival. Those animals which did not respond to tactile stimulation at the mantle fringe were considered dead. Animals surviving the first 24-hour exposure period were retained in the experimental salinities for three additional days, and observation on their condition recorded after each 24-hour period (Table I and Fig. 1).

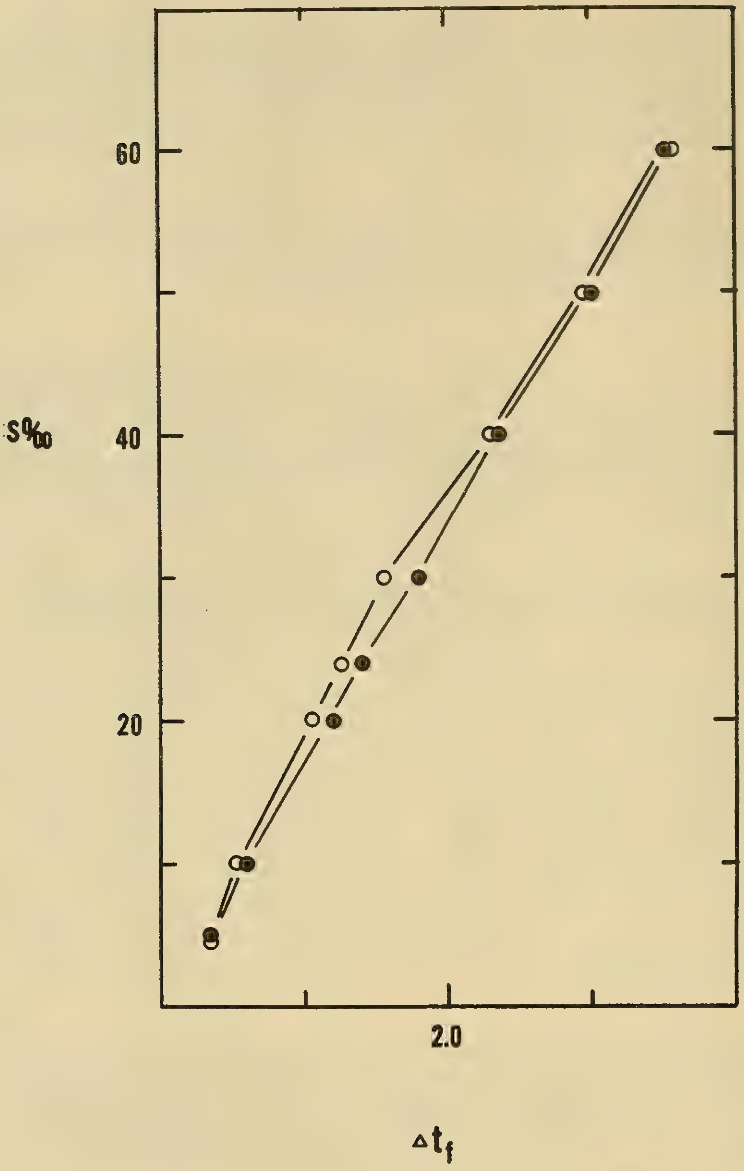

FIGURE 1. Animal-transfer experiments. ○——○ Hemolymph; ●——● sea water.

CROSS-TRANSFER EXPERIMENTS

The sea water in one control tank was diluted gradually from an initial salinity of 24‰ to a final salinity of 12‰ over a period of 24 hours. This was effected by slowly adding fresh water into the tank and assuring complete mixing with a small stirrer. Forty-eight per cent of the animals survived this system of salinity change. The animals were retained in this "50% sea water" for an additional 24 hours, during which time an additional 5% of the animals died. Ten animals were carefully removed from the experimental tank and returned to a control tank (24‰). Ten additional animals in the 12‰ tank were loosened from their attachment sites in order to completely expose their ventral soft parts to the surrounding water. These animals were retained in the 12‰ tank. Animals in the control tank were treated in the same manner, *i.e.*, 10 were transferred into the experimental tank, and 10 were removed from the substrate but retained in the 24‰ tank. After six hours the animals were examined to see if they had reattached to the tank bottoms.

In the hypoosmotic situation, it was observed that the 20 animals which had been entirely exposed to the experimental sea water (12‰) could not reattach. This included 10 animals from the control salinity as well as the 10 animals which had been acclimated to the stress salinity and then lifted up to completely expose the ventral surface. By contrast, all 20 animals which had been completely exposed to the control salinity, whether they had come from the experimental salinity or had been removed from the bottom of one of the control tanks and replaced, retained a complete ability to reattach.

The same sort of experiment as that just described was repeated under hyperosmotic conditions. The salinity of one control tank was slowly concentrated from the initial 24‰ to the final salinity of 49‰, over a one-day period. This was accomplished by slowly adding concentrated artificial sea water to the tank and mixing as above.

The results obtained were essentially identical to those observed under hypoosmotic conditions, *i.e.*, all animals which had their soft parts completely exposed to the experimental sea water showed no ability to reattach, while those animals which were exposed to the control salinity reattached in all cases. The average (of four replicates) per cent survival observed after the 24-hour concentration period was 80%, and an identical value was observed after the additional 24-hour period just prior to cross-transfer.

Activity measurements

Activity of the individual animals in the different salinities was recorded in the following manner: Animals in the control tanks were placed under gradual stress over a 24-hour period until values of 12‰ in the hypoosmotic tank and 49‰ in the hyperosmotic tank were obtained. Animals not firmly attached to the bottom of the tanks were removed; the remaining animals were encircled with a pencil, outlining the shell margin of each animal. All animals were then observed periodically and the percentages which had moved perceptibly from their original positions in the experimental as well as the control tanks were recorded:

S‰	*% activity*
12‰	2.4%
49‰	8.9%
24‰	35.0%

Reattachment after stress

Animals in the control tanks were subjected to sudden osmotic stress by a dilution of the sea water to ½ normal (12‰) or a concentration to twice normal salinity (48‰) and left until a few animals in each tank had become unattached. The salinity in each experimental tank was then readjusted to the normal value and those animals which recovered their ability to attach after the stress was removed were noted. This was done in an effort to investigate any correlation between the ability to attach and the ability to survive. It was found that those animals which had become released from the substrate could not, under any circumstances, recover from the stress after a return to a normal salinity.

Considering the fact that these animals live in an environment where desiccation at low tides may be encountered, experiments were designed to study the responses of *Siphonaria pectinata* to the effects of desiccation, in hopes of determining the maximum period which the animal may withstand these effects under laboratory conditions and still recover when resubmerged in sea water.

Desiccation experiments

Animals were removed from the control tanks and wet weights obtained. The weighed animals were then placed in glass containers with frosted bottoms and the containers with snails submerged in the control tanks for 24 hours to allow for attachment to the frosted glass substrate. The containers were removed from the tank, the water removed, and the dishes with animals attached to the bottom kept at 24° C. Two of the animals were removed from one of the dishes every 6 hours over the next 72-hour desiccation period; one was placed back into the control tank and after 4 hours exposure to the control sea water, the animal was examined and its condition noted. The second animal was weighed, the hemolymph collected, and the Δt_f of the hemolymph determined, as well as the weight change (Fig. 2).

The recovery experiments following desiccation indicate that animals can survive up to 54-hours desiccation, and recover completely when resubmerged. Such animals, upon return to the sea water, regained their initial weights in approximately one hour. Animals exposed to desiccation periods in excess of 54 hours did not recover. It was observed that in such animals, the body had become shrunken to such a great extent that the mantle had been stretched to the point that it had pulled free from its point of insertion along the inner margin of the shell.

Studies on attached animals

These experiments were designed to investigate the osmotic behavior of the hemolymph and salinity tolerance of those animals which had been allowed to acclimate and become attached to the substrate before exposure to sudden or gradual salinity alterations. The experiments were conducted using procedures quite similar to those employed in the experiments involving transfer of animals. In this case, however, the animals were not transferred into the stress salinities; they were left undisturbed and the salinity of the surrounding sea water was altered. The survival averages and Δt_f of the hemolymph will be compared with the values obtained in the animal-transfer experiments.

Experiments were conducted using one 15-gallon tank as a control, and four 15-gallon experimental tanks. Two of the experimental tanks were used for hypoosmotic conditions, and the other two for hyperosmotic studies. All five tanks

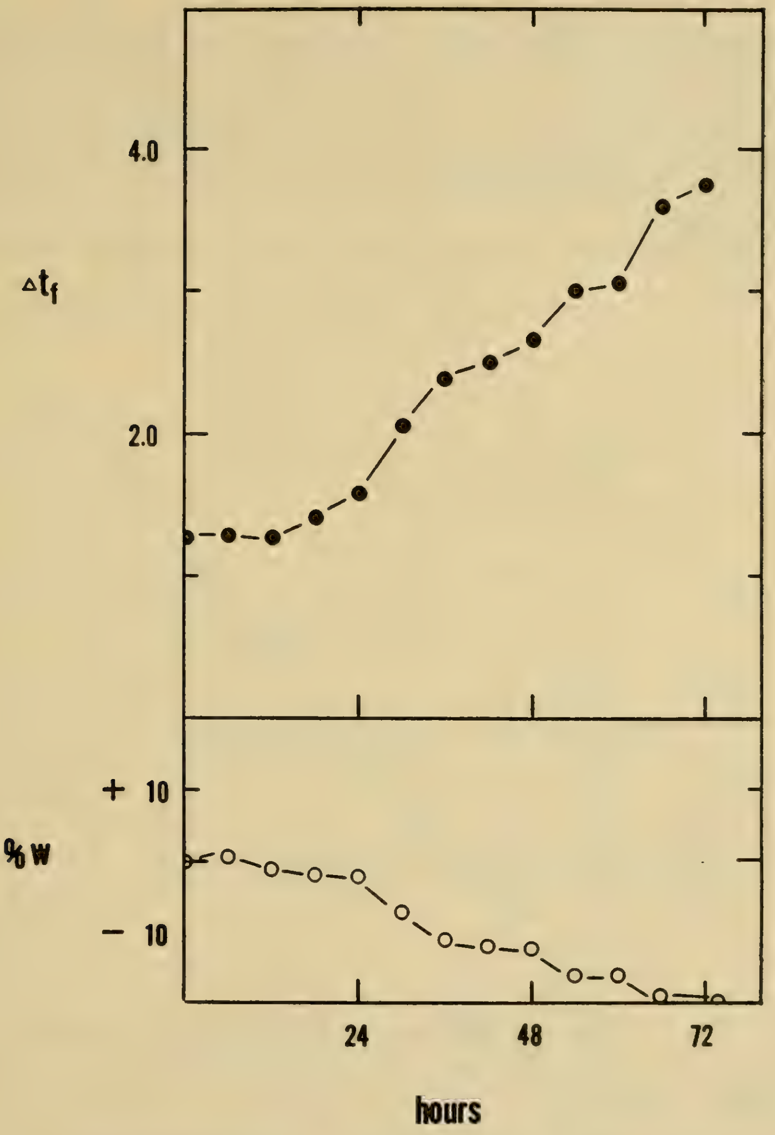

FIGURE 2. Desiccation experiments. ● —— ● Δt_f fluctuations; ○ —— ○ weight fluctuations (%W).

initially contained the same number of animals and were maintained at the control salinity. Seventy-five animals were placed in each tank and allowed to acclimate for several days.

The salinity in two tanks was slowly diluted at the rate of 5‰ per 24-hour period from the initial salinity of 24‰ to a final salinity of 5‰ over a period of four days. Simultaneously, two other tanks were concentrated at the rate of 10‰

per day until a final salinity of 75‰ was reached after five days. The control tank was maintained in the usual manner. Hemolymph and appropriate sea water samples were collected at the end of each day just prior to the next adjustment in salinity in each of the four tanks. Thus four pairs of samples, two of which came from different tanks of identical salinity, as well as a control pair of samples, were collected each day. Measurements of the Δt_f for each sample were conducted. Survival data in the different salinities were also collected daily and expressed as percentages (Fig. 3).

In another experiment, the salinity of two tanks was quickly diluted from the

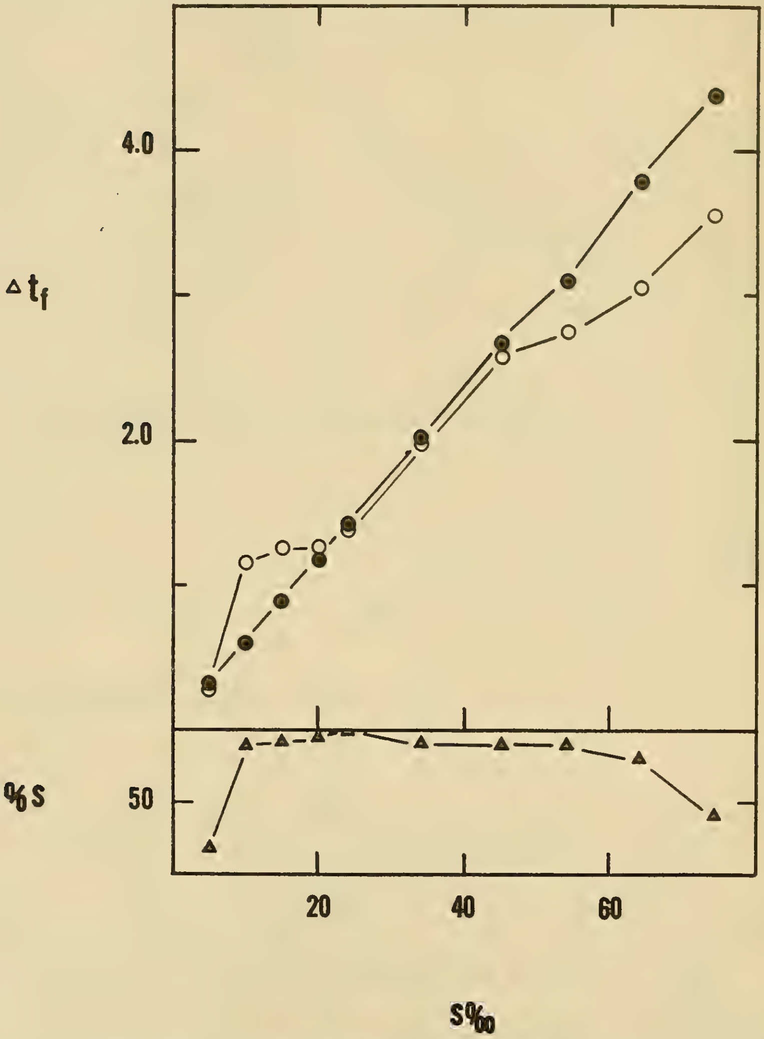

FIGURE 3. Responses of attached animals to gradual salinity change. ○——○ Hemolymph; ●——● sea water; ▲——▲ % survival (%S).

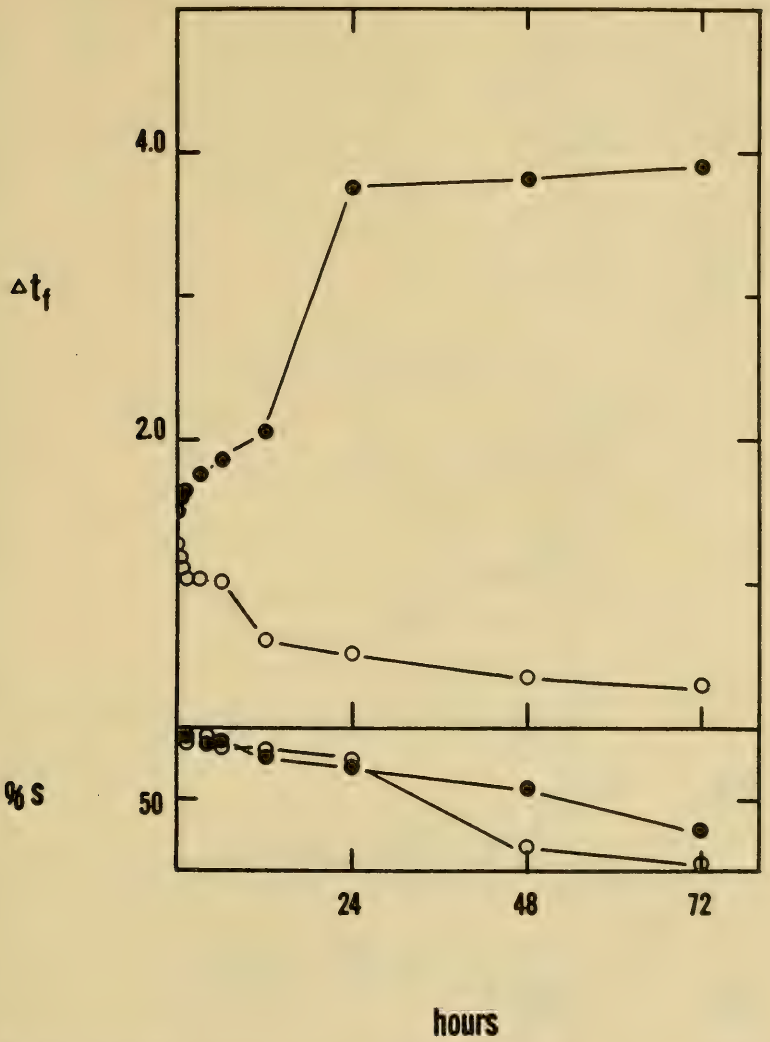

FIGURE 4. Responses of attached animals to sudden salinity change. ○——○ Hemolymph, hypoosmotic tanks; ●——● hemolymph, hyperosmotic tanks; %S = per cent survival.

initial value of 24‰ to a final value of 4‰ by the addition of fresh water. Similarly, two other tanks were quickly concentrated to a value of 74‰ by the addition of a concentrated sea salt solution. The salinity changes were effected as quickly as possible in order to obtain an approximately instantaneous change to a stress condition. After the alterations in salinity, hemolymph samples and a water sample were taken from each experimental salinity at the following time intervals: 0.25, 0.5, 0.75, 1.0, 3, 6, 12, 24, 48, and 72 hours. The Δt_f of the hemolymph and sea water samples were determined and per cent of the animals surviving in each tank at each time period was also recorded (Fig. 4).

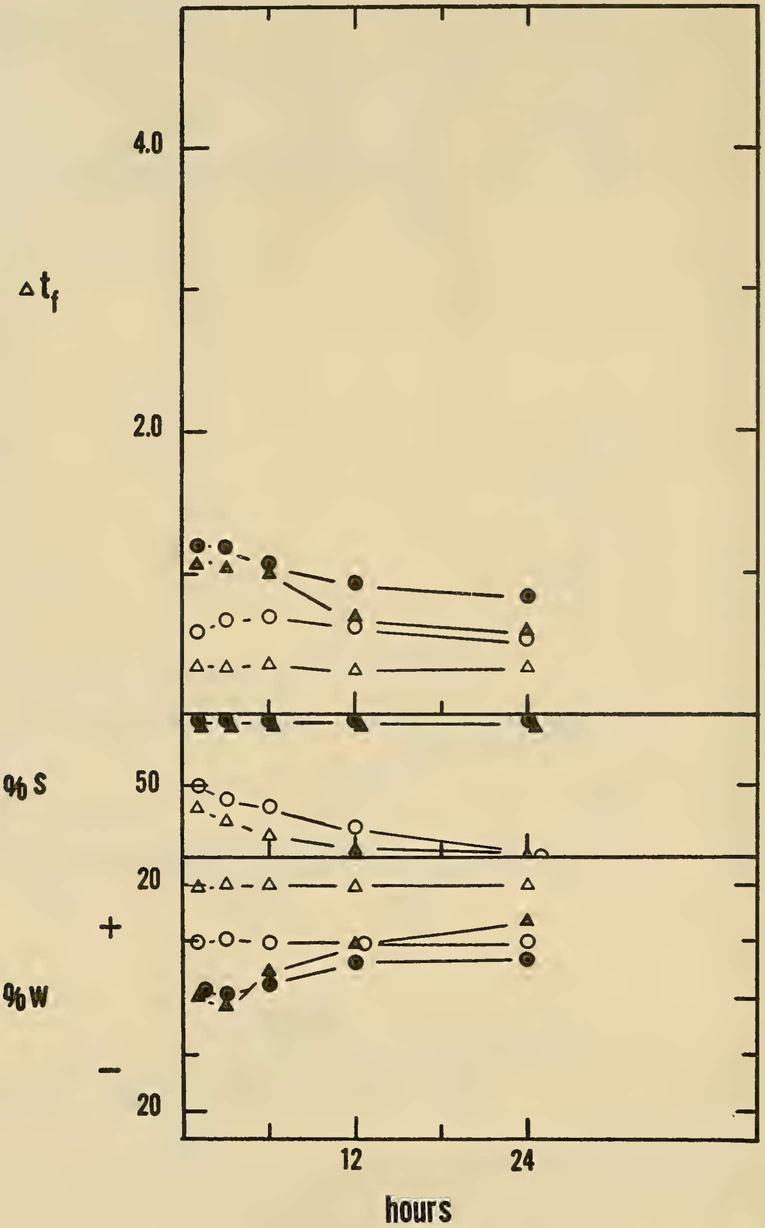

Figure 5. Comparative study—responses under hypoosmotic stress. ▲——▲ Attached animals, 5‰; △——△ unattached animals, 5‰; ●——● attached animals, 10‰; ○——○ unattached animals, 10‰; %W = per cent change in weight.

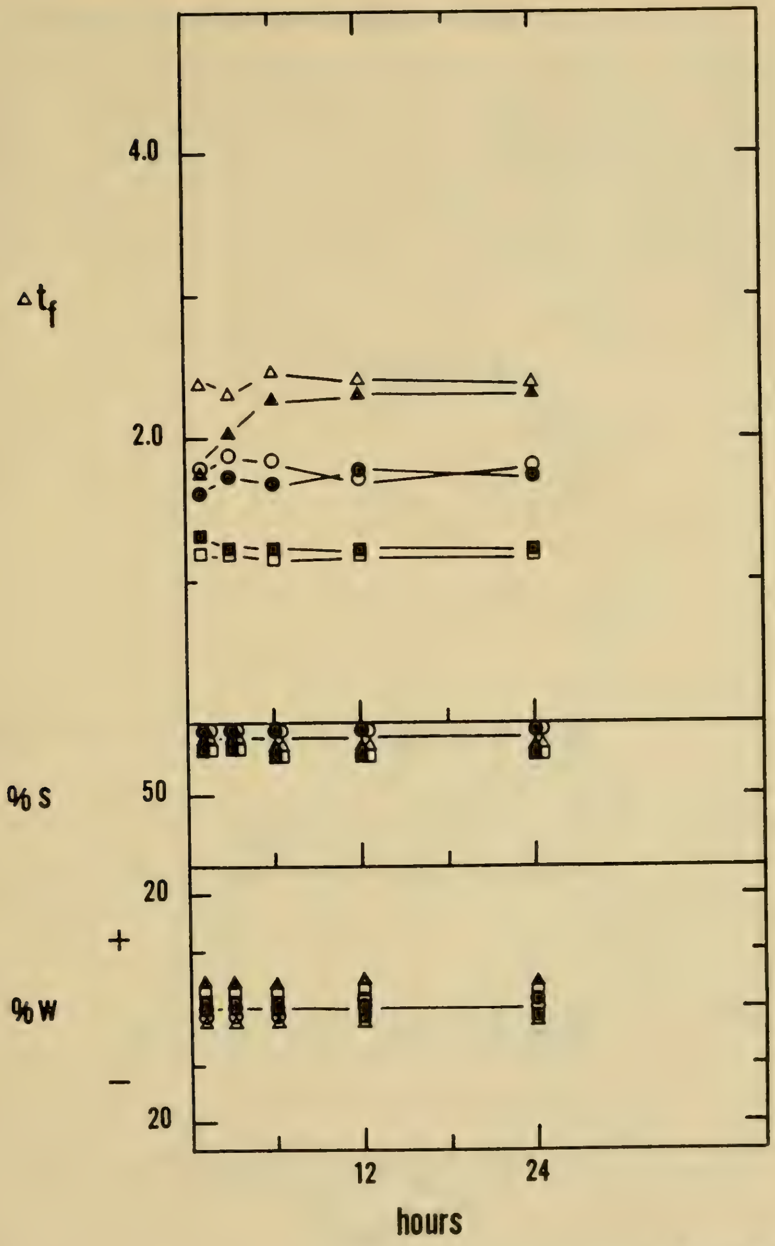

FIGURE 6. Comparative study—responses in the normal range ■——■ Attached animals, 20‰; □——□ unattached animals, 20‰; ●——● attached animals, 30‰; ○——○ unattached animals, 30‰; ▲——▲ attached animals, 40‰; △——△ unattached animals, 40‰; %W = per cent change in weight.

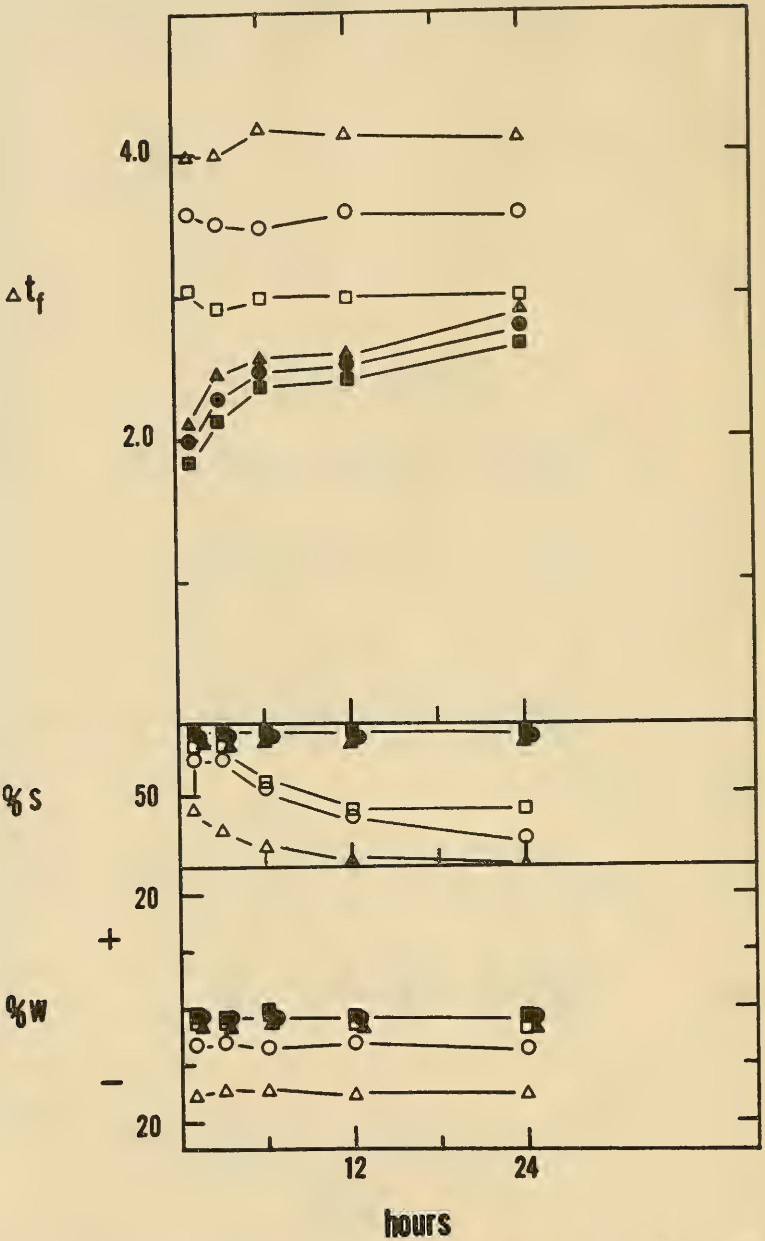

Figure 7. Comparative study—responses under hyperosmotic stress. ■——■ Attached animals, 50‰; □——□ unattached animals, 50‰; ●——● attached animals, 60‰; ○——○ unattached animals, 60‰; ▲——▲ attached animals, 70‰; △——△ unattached animals, 70‰; %W = per cent change in weight.

COMPARATIVE STUDY BETWEEN ATTACHED AND UNATTACHED ANIMALS

Individual animals were weighed and marked and placed in frosted dishes, five animals per dish. The dishes were then placed in one of the five recovery tanks and allowed to acclimate for 72 hours. During this period, animals were observed closely and those which crawled out of the dishes were replaced. After the acclimation period, dishes were removed from the tanks and any accumulated feces were washed out with freshly prepared sea water of the control salinity. The wash solution was poured off, and the dishes and attached animals were blotted dry

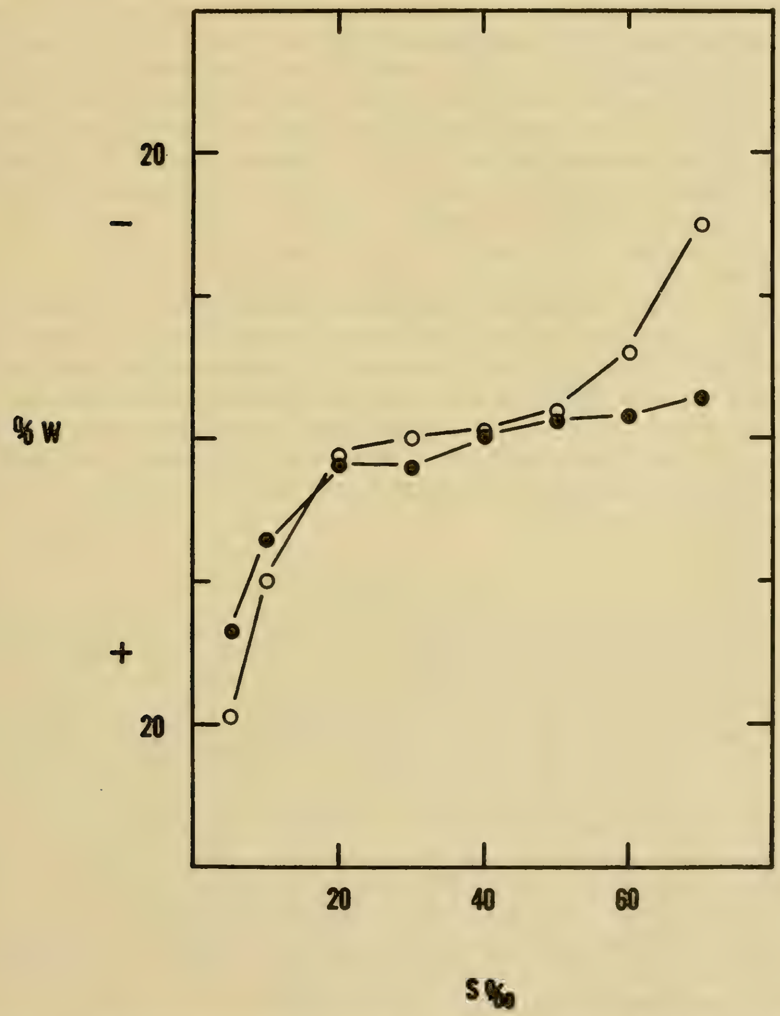

FIGURE 8. Comparative study—comparison of weight fluctuations, attached and unattached animals. ● —— ● Attached animals; ○ —— ○ unattached animals; %W = per cent change in weight.

with absorbent paper. Fifty milliliters of sea water were added to each container. Two containers were used per experimental salinity of: 5, 10, 20, 30, 40, 50, 60, 70‰ and the control salinity, 24‰. To examine the effects of evaporation on the above containers, one blank control dish containing 50 ml. of water and no animals was prepared for each experimental and the control salinity. At time intervals 1 hr., 3 hr., 6 hr., 12 hr., and 24 hr., two animals from each salinity were removed and then hemolymph collected. Samples of the surrounding experimental water were also collected at each time period. The Δt_f of each hemolymph sample of the surrounding medium was determined, as well as the initial and final samples taken from each of the blank control dishes. The remaining set of dishes was left covered for 24 hours, and the five animals in each dish were observed periodically in an effort to detect any swelling, shrinking, or other visible changes in the physiological state during the stress period. After 24 hours, a sample of the surrounding water was taken from each dish and the Na^+ concentration determined; these concentrations were compared to those obtained for the water from the blank control dish containing no animals. The five animals in each of these dishes were then weighed and their final weights compared to the original (pre-stress) weights.

A similar experiment was conducted using glass containers. However, in this experiment, animals were removed from the dishes after acclimation and a small piece of plastic screen was placed in the bottom of each dish. The animals were then placed on this screen and the test solution (50 ml.) added as above. Under these conditions, the animals were submerged in the water but were unable to make contact with the bottom of the dish, making attachment impossible. Thus the complete surface area of the foot and soft parts was exposed to the experimental sea water. Animal weights were recorded just prior to removal of hemolymph samples, at the same time intervals used in the preceding experiment. The results are summarized in Figures 5–8.

DISCUSSION

The data compiled in Figure 1 indicate that *Siphonaria pectinata* has no ability to osmoregulate over the range of salinities tested under the experimental conditions employed. The freezing point depressions for hemolymph and sea water are not considered to be different, since the values represent averages of fourteen determinations with a standard deviation of 0.07 and 0.09 for sea water and hemolymph, respectively. It was therefore concluded that the hemolymph remains essentially isosmotic with the external milieu at all salinities between 5‰ and 60‰.

From the survival data compiled in this experiment, it is proposed that the animal can tolerate salinities between 20‰ and 40‰ with no apparent deleterious effects. This range of salinities will be subsequently referred to as the "normal range." Above and below the limits of this normal range of salinity, it was found that the animals could not reattach to the substrate when placed in the experimental sea water. Furthermore, no animal which was unable to effect reattachment survived for longer than 24 hours after exposure to the sea water above or below the normal range.

A comparison of these results with those found by Harry and Bucher (unpublished) shows a close correlation between the fluctuations in total osmotic pressure and those of the chlorides of the hemolymph in different salinities. It is thus concluded that a measure of the chlorides is a good approximation of the total osmotic behavior of the hemolymph of *S. pectinata* under the defined conditions.

In the cross-transfer experiments under hypo- and hyperosmotic conditions, per cent survival after 24 hours exposure was greatly improved over the results reported in the animal-transfer experiments, which involved a removal of the animal from the substrate. No better than 25% survived after 24 hours exposure to a salinity below the normal range in the animal-transfer experiments; however, in the cross-transfer experiments, in which the animals were placed under stress without the accompanying removal from the substrate, 45% of the animals had survived 12–18 hours of the dilution period as well as 24 additional hours at a constant salinity of 12‰. In the hyperosmotic situation, 80% of the animals survived a similar length of exposure to water considered to be above the normal range of salinity. Furthermore, in both cases (hypo- and hyperosmotic), all the animals which died during the experiment died within 6–12 hours after the salinity had passed out of the normal range. This may be interpreted to mean that those animals which did not survive the initial dilution in the attached state lacked some mechanism for adaptation to the stress salinity which the survivors possessed. Presumably the ability to withstand the initial stress was lost through previous injury.

These results are interpreted to mean that the animals show improved survival if undisturbed from the attached state on the substrate. Furthermore, mere exposure of the soft parts of the body seems to be the key to the reason the animals in the stress salinities could not reattach. The possibility that the animals were injured during removal from the substrate may be safely discarded as a reason for inability to reattach, since all animals in the control tanks, which were treated in the same manner as those in the stress salinities, reattached.

It can be concluded that a short period of exposure of the soft parts (head-foot complex and mantle extension) to an extreme salinity outside the normal range renders the animal unable to reattach to the substrate. It would appear that such a short exposure to the stress salinity would yield a similar effect in those animals which were removed from these salinities and transferred to the control tanks, since they experienced a similar duration of exposure to the stress salinity during transfer. Presumably the immediate and sustained exposure to the control salinity after this brief period of total exposure to the stress solution is sufficient to reverse the effects of the stress salinity.

The preceding results prompted the formation of an hypothesis to explain the discrepancy in survival ability in salinity extremes outside the normal range between attached animals and those which had been completely exposed to the stress salinity. Animals in the hypo- and hyperosmotic salinities were much more difficult to remove from the substrate—so difficult, in fact, that they seemed almost to form a "water-tight bond" between the outer margin of the shell and the substrate. It is thus postulated that attached animals, when faced with an environmental salinity outside the normal range, respond to the stress by active contraction of the foot musculature, effecting a tight union between the shell edge and substrate. It is further proposed that such a "clamping response" of the animal, followed by the maintenance of tonus of the muscles involved, could greatly reduce water flow between the foot and the environment during periods of stress when the osmotic gradient is high due to salinities outside the normal range. Furthermore, previous attachment to the substrate seems to be obligatory, since animals which have been removed from the substrate during periods of osmotic stress show no ability to elicit the proposed response.

Indirect evidence for this hypothesis comes from observations gathered during the animal-transfer and cross-transfer experiments. Animals which had been previously attached showed much less tendency to swell or shrink in hypo- and hyperosmotic stress salinities than did those animals which had been removed from the substrate and completely exposed to the stress salinity.

The results of the activity measurements also support the notion that the animal elicits the clamping response during periods of osmotic stress. The reduced activity observed shows a direct correlation with the proposed response, which is plausible since any active foraging on the part of the animal would tend to sacrifice the tight bond with the substrate which had been established. Further evidence was also obtained from direct microscopic examination of the animals acclimated in hypo-, hyperosmotic and control salinities. Carmine particles were added near submerged animals which had attached to microscope slides. The so-called barrier erected between the mantle fringe-shell complex and the slide appeared near-perfect in the stress salinities; the only bridge across this barrier lay in the region of the pneumostone, where water was observed to be circulating in and out at regular intervals. Except in this region, the particles were not observed to cross the shell margin barriers. By contrast, in the control animals, the red color was seen to pass freely beneath the shell and much of the foot margin acquired a red hue, due to the carmine particles being trapped in the mucus covering the lateral margins of the foot. These observations lend further evidence for reduced ciliary activity and increased muscular contraction in response to the stress salinity.

Those animals which do not show the clamping response and become dislodged from the substrate due to salinity stress show no ability to recover and survive when placed into a salinity of normal concentration. These animals have presumably lost, through injury, the ability to clamp themselves down in response to the stress. If these animals are exposed to the stress for more than a few minutes, they are irreparably injured by the stress medium. This experiment points out the obvious adaptive advantage held by those animals which possess the ability to show the clamping response. Furthermore, it lends evidence to the premise that ability to attach in stress salinities is directly correlated with the ability to survive.

The desiccation experiments indicate that the animals, when attached, could withstand desiccation for up to 72 hours. Furthermore, the limiting factor here seems not to be one of water loss so much as rupture of the mantle itself, that is, mechanical injury. This finding points up the adaptation which *S. pectinata* has acquired for an existence in a zonation along the jetties where the animals might be subjected to periods of desiccation during low tides. It is unlikely that the animal would ever be exposed for longer than 12–18 hours in the Galveston region; however, on a warm day, evaporation could be increased to the point that exposure under field conditions would be more detrimental than those in the laboratory at 25° C.

The next group of experiments was conducted entirely with animals attached to a glass substrate. An effort was made to show the osmotic fluctuations of the hemolymph when attached animals were presented with hypo- and hyperosmotic stress and allowed to show the clamping response. These experiments involved a gradual changing of the concentrations of the sea water to hypo- and hyperosmotic salinities, resulting in the corresponding stress conditions. Examination of the survival data indicates that animals survived much better in the attached state under gradually increasing osmotic stress than was found after instantaneous exposure in the animal-transfer experiments. Assuming the concentration did not reach a stress level in the hypoosmotic conditions until the second day when the salinity reached 20‰, 19% of the animals managed to survive three additional days of increasing stress to a final salinity of 5‰. In the hyperosmotic situation, the salinity was concentrated over a period of five days to a final value of 74‰, after which time 43% of the animals were still alive. This represents a 43% increase in survival over the animals in the animal-transfer experiment, which after exposure to 60‰ survived no longer than 24 hours. Furthermore, per cent survival showed no large decrease in the hyperosmotic tanks until the fifth day, which was 24 hours after the concentration had reached 74‰. This is considered to be significant, since the upper limit of the normal range had been attained 48 hours previously. Therefore, it is concluded that the clamping response is more beneficial to the animals under hyperosmotic conditions than hypoosmotic ones. This does not imply that the clamping response is ineffective in hypoosmotic salinities; it merely hints that it is more effective in allowing the animal to survive salinities above the normal range than below it.

Examination of the data obtained for Δt_f for hemolymph and sea water at the different salinities (Fig. 3) lends further evidence to our hypothesis, *i.e.*, any deviations from the external Δt_f (isosmotic line) by the hemolymph are primarily due to restriction of water movements resulting from the clamping response. The values found for Na^+ fluctuations are not herein reported, for Δt_f and Na^+ concentration were found to be essentially identical as regards increments of deviation from the external milieu. This indicates that measurement of the Na^+ concentration of the hemolymph is directly proportional to the total osmotic concentration at all salinities tested. This conclusion agrees with that proposed earlier concerning hemolymph chloride concentration.

A closer examination of the data composing Figure 3 shows that within the range of 20–45‰, the hemolymph Δt_f values correspond very closely with those of the external milieu. However, the values are markedly higher than the sea water below 20‰, and are lower than the sea water above 45‰. It is further noticed that when the salinity was diluted from 10‰ to 5‰ over an additional 24-hour period, the hemolymph values approximate that of the external milieu. No such drop was observed at the opposite end of the salinity spectrum, after the final salinity adjustment in the hyperosmotic tanks.

When the dilution of the hypoosmotic tank reached a level of 20‰, the animals presumably clamped down, hindering equilibration of the hemolymph with the sea water as the environmental dilution was continued. The same phenomenon apparently has taken place in the hyperosmotic situation. As the level of concentration reached 45‰, the animals elicited the clamping response; thus equilibration was hindered as above. It must be pointed out that the restriction of water flow is not a perfect process; if it were, the hemolymph curves would level off when the clamping response occurred, indicating total blockage of water. However, the curves do not demonstrate this in either the hypoosmotic or hyperosmotic salinities. The rate of change of hemolymph concentration is obviously greatly reduced, but it is by no means stopped completely.

The above values imply that the limits for the tentative "normal range" determined by per cent survival in the animal-transfer experiments, *i.e.*, 20–40‰, should be amended. The animal clamps down at salinities below 20‰ or above 45‰; it is therefore proposed that the range of salinity between 20‰ and 45‰ be designated the normal range for optimum survival. Per cent survival begins to fall off when the salinity passes out of the normal range, at both ends of the curve. Furthermore, there is a good correlation between these curves and the proposed notion that the clamping response works more efficiently in hyperosmotic than in hypoosmotic salinities. This is evidenced by the fact that under hyperosmotic stress, the hemolymph concentration remained markedly below the external concentration throughout the experiment, indicating that the clamping response was still operative. However, after 48 hours exposure to hypoosmotic stress, the Δt_f of the hemolymph suddenly dropped back to equality with the sea water. Furthermore, the animals which were subjected to a salinity of 5‰ were all unattached and swollen; only 19% were alive. This would indicate that the clamping response becomes inoperative when the salinity finally reaches a level of 5‰. The inability to maintain the clamped state may be accounted for in a variety of ways. One explanation would be that salinities below 10‰ are unequivocally lethal to the animal. A more plausible explanation would be that, after 48 hours exposure to a stress salinity (below 20‰), sufficient water had entered to allow the foot and soft parts of the animal to swell to the point that maintenance of muscular contraction was rendered impossible. In this partially swollen condition, the animal could thus no longer maintain the clamped state, and would become released. Thus the tissues would be completely exposed to the environment; the end result would be a return to isosmoticity.

In either case, the curves do substantiate the clamping hypothesis, as well as the notion that the animals are more tolerant to hyperosmotic than to hypoosmotic conditions. The actual mechanism responsible for the loss of the clamping ability in hypoosmotic salinities remains obscure.

Responses to sudden osmotic stress

The survival curves (Fig. 4) show that the attached animals survived sudden hyperosmotic stress reasonably well; after 72 hours exposure, 27% had survived. Furthermore, the plot of per cent survival *versus* time shows an approximately linear decrease. Those animals in the hypoosmotic tanks show near-identical survival values through the first 24 hours exposure; however, at this point, there is a sharp break in the curve and over 50% of the animals died between 24 and 48 hours of exposure at 4‰. In this experiment, any unattached animals were considered dead, due to the earlier findings. Therefore, the initial 5–6% of the animals which had become released due to stress after 30 minutes exposure were presumed to be those which could not effect the clamping response.

The approximate linearity of survival with time in the hyperosmotic salinity is to be expected; it suggests that with increasing exposure, more and more animals pass the salinity tolerance limit, become unattached, and die. Those animals which die first presumably have more imperfections along the margin of the shell. Thus, they are less efficient at restricting water entry than are those animals with more uniform shells which form a more efficient bond with the substrate. This argument is strengthened by the observation that the longer an animal had been maintained in the laboratory before it was used in an experiment, the more efficient was the clamping response. Observations showed that after 2–3 weeks of maintenance in the control tanks, irregularities in the shell edge had been largely repaired through shell regeneration.

The sharp break in the survival curve in the hypoosmotic salinities (Fig. 4) shows a strong resemblance to that observed in the first experiment conducted entirely with attached animals (Fig. 3). It seems probable that this deviation in slope represents the point at which the animals begin to swell to the extent that the clamping response is obstructed.

The relationship between desiccation and osmotic dehydration

It has been shown that the animals which exhibit the clamping response show tolerance to hyperosmotic conditions for periods of 72 hours or longer. Similarly, the desiccation experiments (Fig. 2) indicate a maximum survival time of approximately 54–72 hours at room temperature. In view of these similarities, it appears that desiccation during exposure and water loss due to hyperosmotic dehydration are similar phenomena when faced by *S. pectinata*. This notion is substantiated by the fact that in both cases, the animal exhibits the clamping response. That this response was operative during the desiccation experiments is not obvious from the curve in Figure 2; however, during these experiments the animals were observed to cease all movement and to adhere very strongly to the substrate, two factors characteristic of the proposed clamping response.

The best line of evidence for these two phenomena being essentially identical would be a conclusive documentation that the animal does, in fact, behave as an osmometer. If such were the case, then any weight loss in hyperosmotic salinities could be explained completely in terms of water loss. This would of course be identical to weight loss during desiccation. In keeping with these considerations, weight fluctuations in the different salinities were measured in the course of the experiments involving a direct comparative study between attached and unattached animals' responses to osmotic stress.

The results of these experiments are summarized in Figures 5–8. These experiments were designed to show behavioral differences between attached and unattached animals after sudden exposure to a variety of salinities. Experiments were conducted for only 24 hours in order to obtain a direct comparison between the two groups of animals over a time period considered to be the maximum period which the animal would encounter severe stress conditions in nature. The curves illustrating Δt_f values for attached and unattached animals in Figures 5–8 are essentially self-explanatory. These results reiterate the efficiency of the clamping response in restricting hemolymph concentration changes outside the normal range. Furthermore, since the foot and soft parts of half the animals used were completely exposed to the sea water throughout the experiments, examination of the Δt_f values lends information concerning the length of time necessary for equilibration of the hemolymph with the sea water. In all cases, this equilibrium was established within one hour. This serves to illustrate how permeable to water the foot actually is when completely exposed to the external environment. In the salinities below 20‰ (Fig. 5) and above 40‰ (Fig. 7), the clamping response is seen to markedly restrict this equilibration.

Examination of the survival curves shows that among the attached animals, at least 85% of the animals survived after 24 hours. This is in direct contrast to those animals, which because of the presence of the plastic screen, were prevented from effecting the clamping response. Consequently, survival values for these animals were poor in the salinities above and below the normal range. The fact that all animals in 5‰ and 10‰ were dead after 24 hours, while complete mortality in the hyperosmotic range was observed only in the 70‰ salinity supports further the notion that the animals are more tolerant to hyperosmotic conditions than to hypoosmotic ones.

In all cases of salinities outside the normal range, the weight fluctuations for those animals which were allowed to attach were markedly less than were the fluctuations found in the unattached group. Both the attached and unattached groups were maintained under identical conditions. The weight changes of the unattached animals (Fig. 8) may be considered as ideal behavior for the animal, in the absence of any control factors. The weight fluctuations of the attached group represent the animal as it would behave under normal physiological conditions. In the latter case, it is evident that the clamping response is operative. This is most obvious in those salinities hyperosmotic to the normal range; here, the weight variations have a marked tendency to level off. The discrepancy between weight variations is also evident in the hypoosmotic stress salinities, but the differences between attached and unattached animals are not as pronounced in this case. This is due to the fact that the experiment was conducted for 24 hours. It has been demonstrated that in some cases 24 hours is sufficient time for the slow inward movement of water to cause sufficient swelling of the foot to nullify the clamping response, depending on the degree of irregularity in the shell margin.

An examination of the weight fluctuations of the unattached animals (Fig. 8) shows the curve produced to be asymptotic about the acclimation salinity of 24‰. Furthermore, little change in weight is observed between the salinities of 20‰ and 40‰. This is approximately the same range as the normal, or "non-stress" salinity range defined earlier.

If the animal in question were behaving as a perfect osmometer, *i.e.*, permeable to water but impermeable to salt, the volume of osmotically active water would be a constant at all salinities. This is based on the assumption that given any increment of change in external concentration, there will be a corresponding proportional increment in weight change observed.

One method for demonstration that an animal is behaving as an osmometer would be to show that the product of the osmotic pressure of the body fluids and the water content of the body is a constant at all salinities. Assuming a constant weight of the animal is non-water in all salinities, it follows that, if weight fluctuations were due solely to water movements, the product of the weight of the animal and the osmotic pressure of the body fluids should be constant.

Application of this method for determination of mobile water yields non-constant values for the different salinities in the case of *S. pectinata*. However, the possibility that the animals are behaving as osmometers outside the normal range cannot be ruled out. Within the normal range of salinity, the animals show little predictability regarding weight fluctuations. Such a wide range of salinity tolerance in the unattached state may possibly imply that some sort of ionic regulatory mechanism is present and operative within the normal range. In the stress salinities, however, the animal shows fairly consistent and predictable weight changes.

It has been shown that water movements do occur between tissues and external milieu in response to salinity gradients; the clamping response acts as a mechano-regulatory aid to the animal in restricting this flux. Any deviation from a theoretical value for mobile water calculations bears no direct relation to the clamping response, although the possibility of an internal ion regulatory mechanism which would complement this mechanical mechanism for regulation would certainly be advantageous and cannot be ruled out.

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RESPONSES OF AN ESTUARINE POPULATION OF THE POLYCHAETE NEREIS LIMNICOLA TO OSMOTIC STRESS

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Several species of the polychaete family Nereidae have been subjects for studies concerning osmotic and ionic regulation (see reviews by Krogh, 1939; Oglesby, 1965a, 1965b, 1968; Potts and Parry, 1964; Smith, 1959). These studies have characterized osmotic responses, and have attempted to determine the mechanisms responsible for the ability of certain of these worms to be hyperosmotic when adapted to low salinities. Among the most important mechanisms which have been hypothesized are: (1) an active transport of salts by the body surface, from the medium to the body fluids; (2) reduction of the permeability of the body surface to salts, or to water, or to both; and (3) production by the paired segmental nephridia of a urine hypoosmotic to the blood and coelomic fluid. It has so far not been possible to detect hypoosmotic urine in any polychaete, and so this remains an attractive, but unsupported, hypothesis. The present report provides data relevant to the first two hypotheses.

In an earlier study, Oglesby (1965a) investigated several steady-state parameters of osmotic regulation in four species of marine and estuarine nereid polychaetes. Three of the four species, *Nereis* (*Hediste*) *limnicola* Johnson (formerly called *Neanthes lighti* Hartman) and *Laeonereis culveri* (Webster), both from fresh and oligohaline waters, and *Nereis* (*Neanthes*) *succinea* Leuckart, from oligohaline to polyhaline waters, were found to be hyperosmotic to the external medium when in low salinities (lower than about 200 m*M* Cl^-, or 36% of the International Normal Sea Water with 560 m*M* Cl^-). The fourth species, *Nereis* (*Nereis*) *vexillosa* Grube, from polyhaline and marine waters, was essentially isoosmotic in all salinities in which it could survive. Long-term survival in the laboratory was restricted to salinities above about 200 m*M* Cl^-. Important differences among these related species have been found in the relationship of the water and chloride concentrations of entire worms to salinity, and in the critical low salinity at which hyperosmotic regulation begins to break down. These differences have a direct correlation with the habitats of the worms.

Studies of water movements under the imposed osmotic stresses of transfers from one salinity to another showed that *L. culveri* had a lower net water movement after a given salinity transfer than either *N. succinea* or *N. vexillosa* (Oglesby, 1965b). Studies by Smith (1957, 1964) indicated that *N. limnicola* had a lower net water gain than did *N. succinea,* after comparable transfers. The studies with *N. limnicola* did not include transfers from low to high salinities. In general, the more euryhaline polychaete species have the lower net water movements in response to osmotic stress (review: Oglesby, 1968).

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Studies of salt movements in these species have been approached in two ways: measurement of total salt fluxes with radioisotope tracers after adaptation to various salinities, and measurement of net salt movements (gain or loss) during the osmotic stress caused by transfers (review: Oglesby, 1965b, 1968; Potts and Parry, 1964). Such studies indicate that the more euryhaline (or homoiosmotic) a species is, the less the salt movements are. In particular, radioisotope studies have shown that chloride fluxes are much lower in *N. limnicola* than in either *N. succinea* or *N. vexillosa.* Osmotic stress studies similarly have shown that net chloride movement is reduced in *N. succinea* after adaptation to low salinities (Oglesby, 1965b). These results suggest (1) that *N. limnicola* is either less permeable to salts than the less euryhaline species, or it has a reduced area of permeable body surface, and (2) that worms adapted to salinities in the osmoregulatory range (salinities below about 36% sea water, in which the worms are hyperosmotic) have a reduced permeability to salts as compared with the permeability of the same species adapted to high salinities.

At the time these studies were under way, it was not possible to extend the osmotic stress experiments to *N. limnicola.* The population of that species used for research purposes was in the fresh-water Lake Merced in San Francisco, California. During the study, this population was drastically reduced, apparently by rotenone used in a routine fish-poisoning program (Oglesby, 1964). The availability of a large population of *N. limnicola* in a tidal creek in Oregon has now permitted study of salt and water movements in this species after salinity transfers, comparable to those earlier experiments with *N. succinea* and *N. vexillosa.* The present report also compares the osmoregulatory ability of this estuarine population of *N. limnicola* with that of the fresh-water population previously studied. A preliminary account has been published (Oglesby, 1966).

Ecological Observations

Nereis limnicola was collected about one-half mile upstream from the mouth of Schooner Creek, a small tidal stream which empties into the estuary of the much larger Siletz River at Taft (a part of Lincoln City) on the central Oregon coast (see map, Fig. 1). This site was investigated on the basis of a preliminary ecological survey of lower Schooner Creek (Squier, 1960), which reported nereids living at low salinities. There are few published records for *N. limnicola* in Oregon. Haertel and Osterberg (1967) found *N. limnicola* near the mouth of the Columbia River, apparently in fresh water. The species is also present in a drainage ditch entering Kentuck Inlet, one of the arms of Coos Bay in southern Oregon (R. I. Smith, pers. comm.). Presumably this is the population referred to by Hartman (1959). It is likely that *N. limnicola* is present in other Oregon estuaries, as it has a reported range from central California to southern British Columbia (Oglesby, 1965a).

Worms were found in stabilized muddy sand on banks and sand bars of Schooner Creek, below the level of a stand of the cord-grass *Spartina foliosa* Trin., and above the water level in the creek at low tide, a vertical distance of about a foot. The worm habitat is exposed at low tide; during this time, worms are found in their galleries 2–6 inches below the surface. Worms were readily available at any time of the year, although occasional high creek flow in winter made collecting

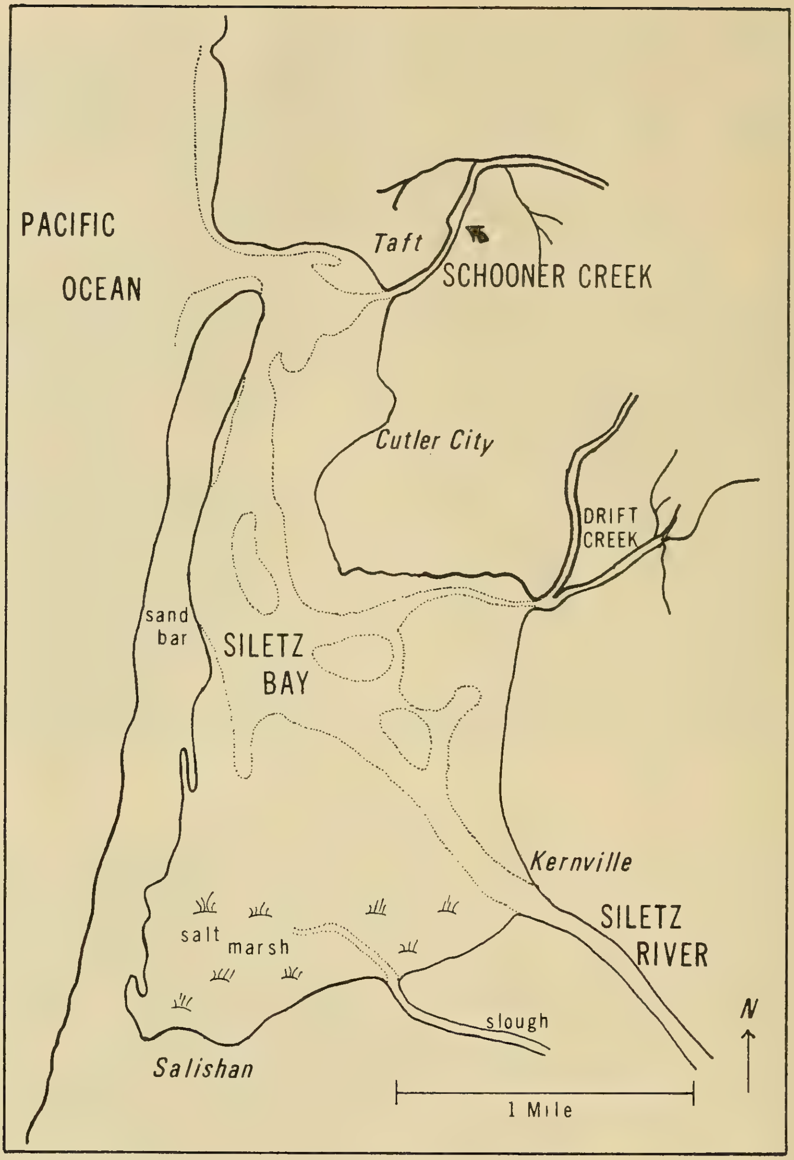
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FIGURE 1. Map of Siletz Bay, Oregon, showing collecting site for *Nereis limnicola* (black arrow). Dotted lines outline water limits at low tide. Adapted from Squier (1960).

difficult. Large worms (weight greater than 100 mg.) could be found at any time, although they were more numerous in autumn.

An attempt was made to characterize the salinity range to which this population of *N. limnicola* is exposed both during a tidal cycle and during the year. Figure 2 shows the chlorosity in m*M* Cl^-, over a two-year period, of seep water (= interstitial water) in the holes from which worms were dug, and of bottom water in the middle of Schooner Creek at low tide, water to which the worms are not directly exposed at the time of sampling. Interstitial water at low tide is always of a higher salinity than creek water.

According to Burt and McAlister (1959), Siletz Bay is typical of Oregon estuaries in showing relatively little mixing between entering fresh water from the Siletz River (the major source of fresh water in this estuarine system), and sea water from the Pacific Ocean entering through the narrow mouth of the bay. This means that the salinity of bottom water of the estuary is considerably higher than that of surface water, which may be nearly fresh. The range of salinities found in the bay varies during the year, due to the great variation in flow of the Siletz River, which dominates the estuary system. Schooner Creek has a fairly low and constant flow throughout the year, varying less in volume than most other

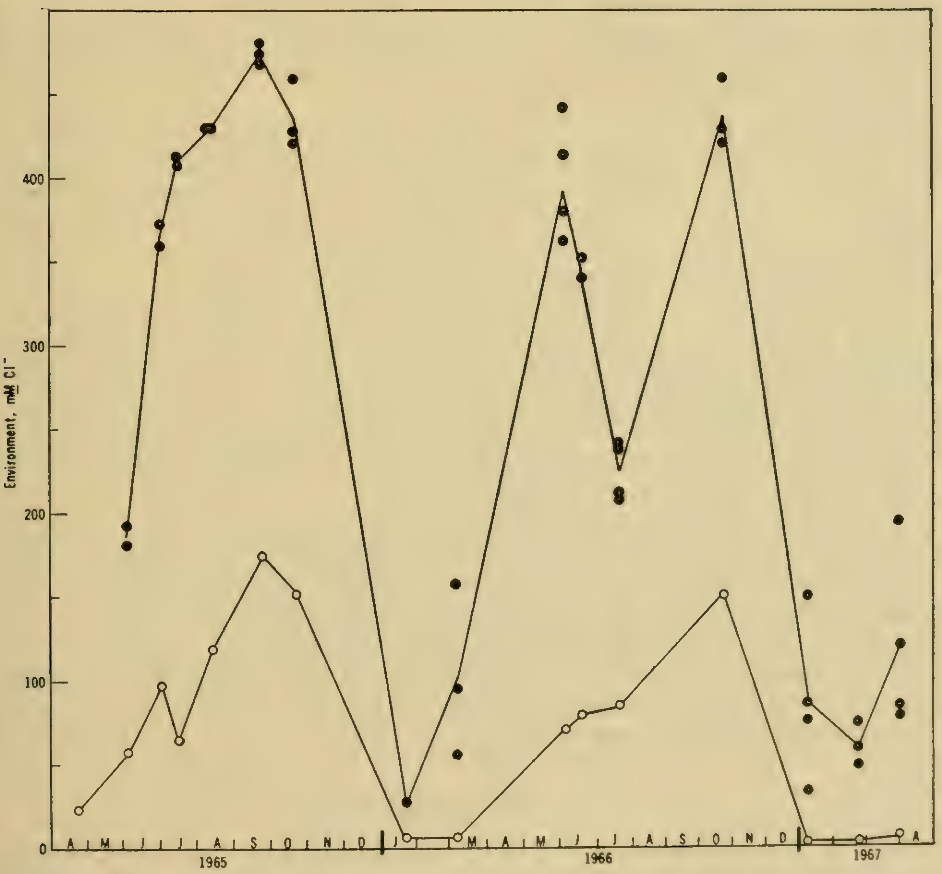

Figure 2. Chloride concentration of habitat of *Nereis limnicola* at Schooner Creek, Oregon, in 1965–67. Solid circles indicate values for seep water into holes dug for worms; open circles, values for bottom water in the middle of Schooner Creek at low tide, adjacent to worm diggings.

coastal streams in the area. This is apparently the consequence of its watershed having been little subject to logging operations. The small volume of flow in the creek means that it has little influence on the salinity distribution in Siletz Bay. At low tide, all the water in the lower reaches of Schooner Creek is derived from further upstream, and flows at such a rapid rate that there is no salinity stratification in the stream. At times of high creek flow, this water is fresh or nearly so (Fig. 2).

At the collecting site, the only times the worms are covered by water is at high and mid tides; they are not exposed to the low-salinity water in Schooner Creek at low tide. Average tidal range is about six feet, and at high tide much water enters the lower reaches of the creek from the bay. The high salinity found in the interstitial water along the banks is probably representative of the more saline bottom water of Siletz Bay, entering as a wedge under the fresher water in the creek. Apparently during a single tidal cycle, the worms are not exposed to the lowest salinities found in Schooner Creek, but only to higher salinity bottom water at high tide, derived from Siletz Bay. This situation is similar to that described by Smith (1956) for the habitat of the closely related *Nereis* (*Hediste*) *diversicolor* Müller in the Tamar Estuary of England.

Seasonally, however, the salinity of the bottom water varies markedly, as reflected in the great seasonal variation in interstitial water salinity (Fig. 2), because of the varying influence of the Siletz River. In winter and early spring, when Siletz River flow is maximal, low salinities are found in the bottom water of Schooner Creek, even at high tide. Much higher salinities are found in bottom water in late summer and early autumn when river flow is minimal.

The distribution of *N. limnicola* in Schooner Creek extends upstream from the area just described. A number of worms have been found at the mouth of a drainage ditch one-half mile further upstream, in an area still subject to tidal influence. No salinity determinations were made in this area, but the aspect of the streamside vegetation is that of a fresh-water habitat, in marked contrast to the salt-marsh vegetation at the usual collecting site. Presumably these upstream worms are never exposed to very high salinities.

The ecology of *N. limnicola* has been described in detail for only one other estuarine location, the lower Salinas River of central California (Smith, 1953). The hydrographic characteristics of the two estuaries are quite different, and none of the locations discussed by Smith is comparable in salinity regimen to the Schooner Creek area. This Schooner Creek population of *N. limnicola* has a habitat with respect to salinity very different from the population in permanently fresh-water Lake Merced previously studied (Johnson, 1903; Oglesby, 1965a, 1965b; Smith, 1957, 1959, 1963, 1964).

Laboratory Methods

Worms were maintained in the laboratory at 10° C. in small covered trays, in a layer of coarse sand covered by 2–3 cm. water of the appropriate salinity. Worms were adapted to different concentrations by graduated changes over a period of time, and were adapted at least a week in the test salinity. Experimental media were made with sea water taken from the open coast and diluted to the desired concentration with spring water (< 1 mM Cl$^-$). The chloride concentrations of the various media were determined by direct titration.

Chloride titrations were performed amperometrically with a Cotlove chloridometer (Cotlove, Trantham and Bowman, 1958). Total body chloride was determined by digesting an entire worm in the chloridometer acid titration reagent (0.1 M HNO_3, 1.75 M acetic acid) at 80° C., and titrating the digest directly. Coelomic fluid samples were taken with 0.01-ml capacity isodiametric micropipets (Kensington Scientific Corp.). These were centrifuged, and the cell-free portion discharged into vials of the chloridometer reagent for titration. The length of the fluid in the micropipets is proportional to the volume.

The day before an experiment, healthy and non-reproductive worms were gently removed from their sand burrows and placed individually in 30–40 ml. of medium of the same salinity, but lacking sand, to permit evacuation of the gut. At the beginning of the osmotic stress experiments, worms were removed from the adaptational medium, dried briefly on paper towels, weighed to the nearest 0.1 mg. on a Mettler Type H analytical balance, and placed individually in 50-ml. beakers with 40 ml. of the stressing salinity, at 10° C. At timed intervals, 10 worms were removed from the stressing medium, rapidly rinsed twice in de-ionized water to remove adherent external chloride, dried, reweighed, and placed individually in volumetric flasks of the chloridometer reagent for digestion and titration of total body chloride. Worms were handled minimally.

Experimental Results

A. *Chloride concentration of the coelomic fluid*

Data on the chloride concentration of the coelomic fluid of *Nereis limnicola* collected from Schooner Creek and adapted to 10° C. are presented in Figure 3. For purposes of ready comparison, regression lines are included, representing data on coelomic fluid chloride and osmotic concentrations for populations of *N. limnicola* from Lake Merced and the estuaries of Walker Creek and Salinas River, all in California (data of Smith, in Oglesby, 1965a, Fig. 2). These worms were maintained at 14–18° C. Also included are data from Smith (1957, Table I) for *N. limnicola* from the Salinas River, maintained at 5° C.

The Schooner Creek population of *N. limnicola* has a pattern of regulation of the chloride concentration in the coelomic fluid not markedly different from the pattern shown by the other populations of this and related nereid species. By inference one can assume that the osmotic concentration of the coelomic fluid, when expressed as the molarity of a NaCl solution with the same osmotic pressure, corresponds to the chloride concentration, as has been found in both *N. limnicola* and *N. diversicolor* (Hohendorf, 1963; Smith, in Oglesby, 1965a; see also Oglesby, 1968). At external concentrations above about 200 mM Cl$^-$, the coelomic fluid is isoosmotic with the external medium. At lower external concentrations, the coelomic fluid is hyperosmotic, down to a critical low salinity at which the internal Cl$^-$ (and osmotic concentration) begins to drop markedly.

There are two points of possible difference between the Schooner Creek worms and those populations previously studied. The critical low salinity appears to be slightly higher than in the California populations. However, no determinations were made for Schooner Creek worms adapted to media between 0.3 and 20 m*M* Cl⁻, and so this apparent difference may reflect only a lack of data. The level of hyperosmotic regulation is somewhat lower in the Schooner Creek worms than in worms from fresh-water Lake Merced (dotted regression line in Fig. 3). A

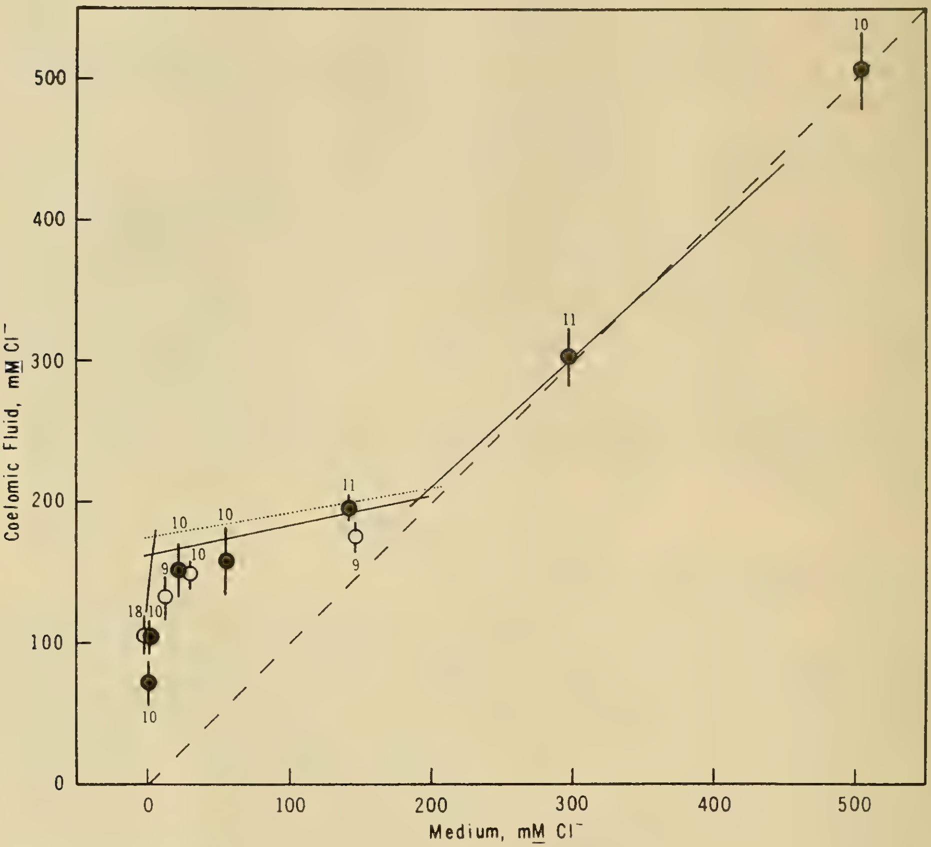

FIGURE 3. Relationship of chloride concentration in coelomic fluid of adapted *Nereis limnicola* to environmental chloride. Solid circles, values for worms from Schooner Creek, Oregon (N = 72; T = 10° C.); open circles, values for worms from Salinas River estuary, California (N = 46; T = 5° C.) (data from Table I of Smith, 1957); solid lines are regression lines for worms from Lake Merced, Walker Creek, and Salinas River estuary, California (N = 339; T = 14–18° C.) (data of R. I. Smith, taken from Oglesby, 1965a); dotted line is regression line for worms from Lake Merced only. Numbers indicate sample size. Vertical bars represent one standard deviation above and below the mean. Dashed diagonal line is line of equal internal and external chloride concentration.

consistently higher level of hyperosmotic regulation in this fresh-water population has been remarked upon before (Oglesby, 1965a; Smith, 1959, 1963), but the difference does not have statistical significance. Smith (1955) found even greater differences in the level of hyperosmotic regulation between spring and autumn samples of a single population of *N. diversicolor* at Kames Bay, Millport, Scotland, than have been found so far between any two populations of *N. limnicola.*

Another factor which may be influencing the level of hyperosmotic regulation is temperature. Smith (1957) has found that the level of hyperosmotic regulation in *N. limnicola* decreases slightly with decreasing temperature. Schooner Creek worms were adapted to a lower temperature (10° C.) than the worms which provided the data for the regression lines in Figure 3 (14–18° C.), and therefore might be expected to have a slightly lower level of hyperosmotic regulation. As shown in Figure 3, there is little difference in the curves at the lower end of the salinity range of hyperosmotic regulation between worms from Schooner Creek at 10° C., and those from the Salinas River at 5° C.

With the data presently available, it is not possible to determine whether the lower level of hyperosmotic regulation found in Schooner Creek worms, as compared with the population in fresh-water Lake Merced, is due to long-term interactions with different habitat conditions, or to differences in maintenance temperature in the laboratory, or even if the difference has real significance.

B. *Chloride concentration of whole worms*

Since the chloride flux determinations derived from the osmotic stress experiments are based on analyses of the chloride content of whole worms, it is important to know how this chloride content varies with external salinity, and whether there are differences in this steady-state parameter among the several populations of *N. limnicola.* This information is presented for Schooner Creek worms in Figure 4. Regression lines representing the data on Lake Merced worms (Oglesby, 1965a) are included for direct comparison of the two populations. (It should be noted that there is an error in Fig. 4 of Oglesby (1965a): the ordinate of that figure should read "Body chloride, μM Cl^-/gm.")

In all except the lowest salinities, there is no difference in the chloride contents of whole worms from estuarine and fresh-water habitats. The drop of chloride content in some samples of worms adapted to external concentrations below 30–40 mM Cl^- may reflect a higher critical low salinity in the estuarine population, as suggested also by the data on coelomic fluid chloride concentration (Fig. 3). Again, more data in this important salinity range (0–50 mM Cl^-) are needed to characterize adequately the adapted responses of these worms to low salinities.

It can be concluded that, in the range of salinities covered by the present osmotic stress experiments (17–504 mM Cl^-), there is little difference between the estuarine and fresh-water populations of *N. limnicola.*

C. *Chloride and water movements during osmotic stress*

The results of the osmotic stress experiments are graphed in Figures 5–7. The change in body chloride concentration, in μM Cl^- per gram wet weight of worm (upper curves in Figs. 5–7), and the change in weight (= change in water volume), in mg. H_2O per 100 mg. wet weight (= per cent change in weight from initial weight) (lower curves in Figs. 5–7) were both measured directly. The change in body chloride concentration which could not be accounted for solely on the basis of water movement could then be calculated. This represents a net

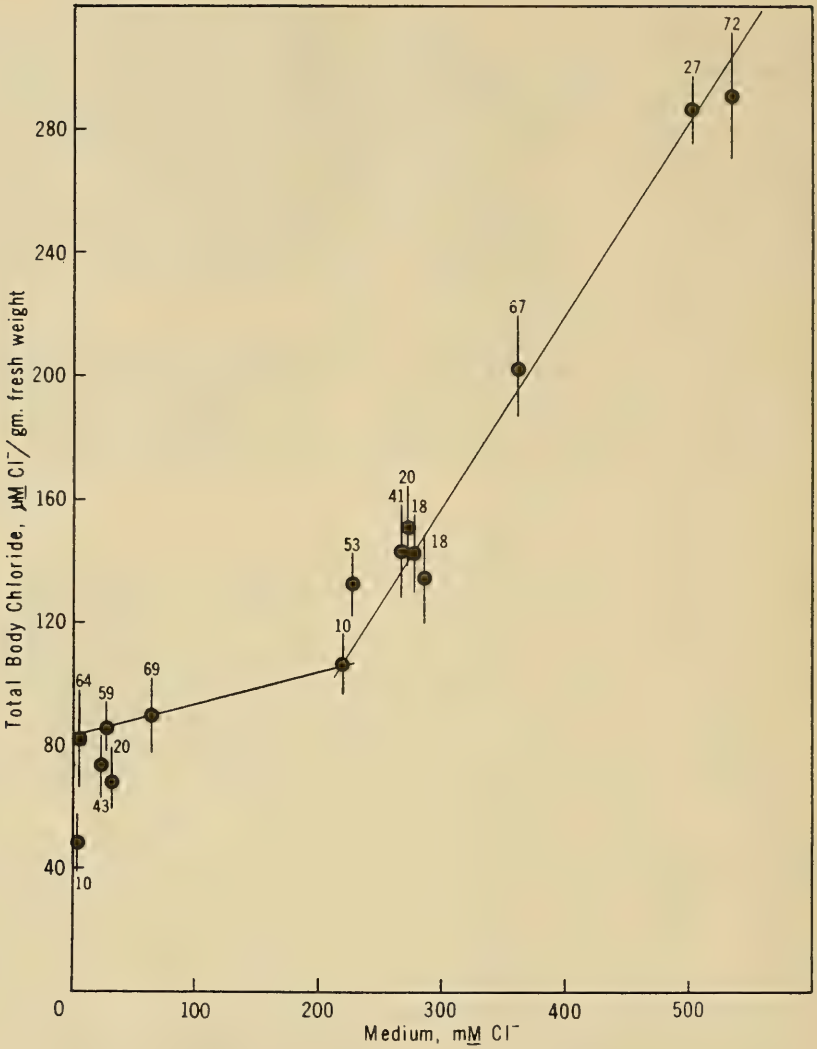

FIGURE 4. Relationship of chloride concentration in entire *Nereis limnicola* to environmental chloride. Points are means for worms from Schooner Creek, Oregon (N = 591). Numbers indicate sample size. Vertical bars represent one standard deviation above and below the mean. Solid lines are regression lines for worms from Lake Merced, California (N = 425) (taken from Fig. 4 of Oglesby, 1965a).

chloride gain or loss down the imposed chloride concentration gradient, and is expressed as μM Cl⁻ gained or lost per gram initial wet weight of worm (middle curves in Figs. 5–7). From the data on steady-state chloride and water contents (Oglesby, 1965a, and regression lines in Fig. 4), the predicted chloride and water contents were calculated for worms fully adapted to the initial and final salinities (see Oglesby, 1965b, for explanation of method). These predicted values are indicated in Figures 5–7 by heavy black arrows. Net inward movement of water and chloride is arbitrarily designated as +, and net outward movement as − in these figures.

1. Downward transfer within the salinity range of osmotic conformity. Nereis limnicola, after adaptation to 90% sea water (504 m*M* Cl⁻), was transferred to 48% sea water (267 m*M* Cl⁻) (two experiments). The concentration gradient set up between internal and external fluids was 237 m*M* Cl⁻ (from Fig. 3), which would be reduced to zero after full adaptation to the new salinity. The results (an average of 17 worms per point) are graphed in Figure 5a. As found in comparable transfers with *N. succinea* and *N. vexillosa* (Oglesby, 1965b, Figs. 1a and 2), there was an immediate decrease in the chloride concentration (μ*M* Cl⁻ per gram wet weight) (upper curve). By the eighth hour after transfer, the chloride concentration of the experimental worms was not statistically different from that expected in worms fully adapted to 48% sea water (black arrow). *N. succinea* and *N. vexillosa,* exposed to a somewhat larger salinity transfer, reached equilibrium with the new salinity after 2–4 hours. Simultaneously there was an uptake

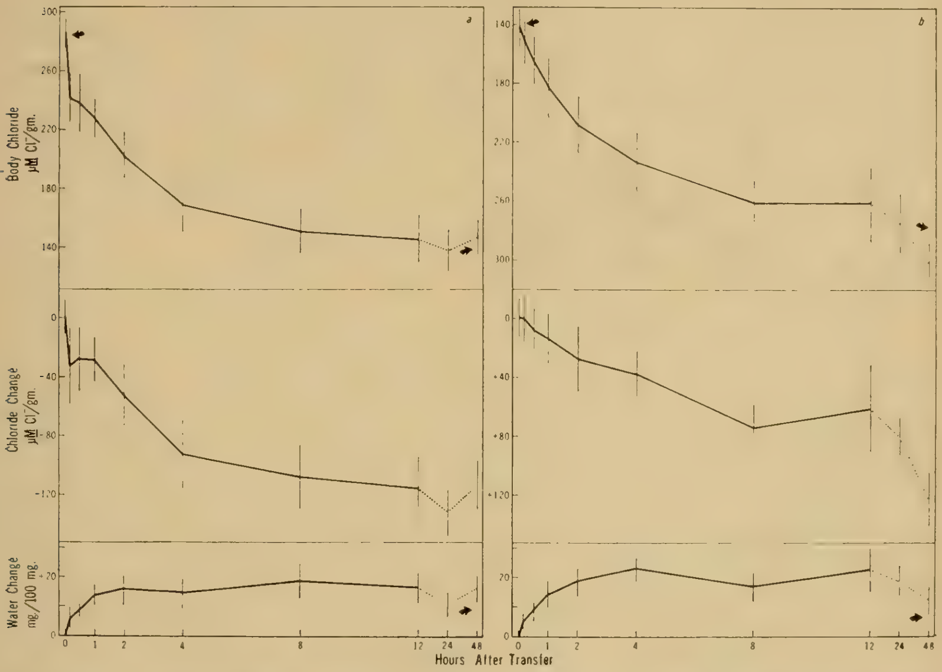

FIGURE 5. *Nereis limnicola* transferred within the salinity range of osmotic conformity. Upper curves: change in chloride concentration of whole animal; middle curves: calculated net gain (+) or loss (−) of chloride; lower curves: weight (water) gained or lost. Vertical bars represent one standard deviation above and below the mean. Heavy black arrows indicate predicted initial and final values for chloride and water concentrations of whole animals. (a) Transfer from 504 m*M* Cl⁻ to 267 m*M* Cl⁻ (N = 172). (b) Transfer from 274 m*M* Cl⁻ to 495 m*M* Cl⁻ (N = 165).

of water (lower curve), with an indistinct maximum also at eight hours. This maximum value was not markedly different from the water content expected of worms fully adapted to 48% sea water (black arrow). A small amount of volume reduction occurred after 12 hours. The appearance of the volume curve is different from the volume curves for *N. succinea* and *N. vexillosa* (Oglesby, 1965b, Figs. 1a and 2). The latter two species gained much more than twice as much water in about the same period of time, and volume regulation was more pronounced, probably because of the great difference between the maximum weight attained after the transfer and the adapted weight.

A portion of the decrease in body chloride concentration cannot be attributed to dilution through water gain. This must be due to a net chloride loss (middle curve), which had a maximum rate of -56 μM Cl^-/gm.-hr., in the first half hour, and an average rate for the entire first hour of -28 μM Cl^-/gm.-hr. This latter rate is less than the maximm loss measured over an hour's time, in both *N. succinea* (-45 μM Cl^-/gm.-hr.) and *N. vexillosa* (-32 μM Cl^-/gm.-hr.) (Oglesby, 1965b). In all three species the chloride concentration change was due not only to water gain along the imposed osmotic pressure gradient, but also to a simultaneous chloride loss along the imposed diffusional gradient. *N. limnicola* had lower rates of both water and chloride movement than the two less euryhaline species.

2. Upward transfer within the salinity range of osmotic conformity. *N. limnicola,* after adaptation to 49% sea water (274 mM Cl^-), was transferred to 88% sea water (495 mM Cl^-) (two experiments). The concentration gradient set up between internal and external fluids was 221 mM Cl^-, which would be reduced to zero after full adaptation to the new salinity. The results (an average of 17 worms per point) are graphed in Figure 5b, with the ordinates reversed in sign for ease of comparison with Figure 5a. This transfer was the reverse of that just described: the directions of the imposed concentration gradients were reversed, but the magnitudes remained the same. There was an immediate increase in the body chloride concentration, approaching the new adapted value (black arrow) by eight hours, caused by both a water loss and a net chloride gain. The maximum rate of chloride gain was $+15$ μM Cl^-/gm.-hr., in the first half hour; the average rate over the first hour was $+13$ μM Cl^-/gm.-hr. This latter rate is much less than the maximum found in *N. succinea* after a comparable transfer ($+60$ μM Cl^-/gm.-hr.) (Oglesby, 1965b). Water loss in *N. limnicola* was not as great as in *N. succinea;* volume regulation was evident after 12 hours. The changes in concentration observed between 24 and 48 hours are not readily explained, but do indicate that even after 48 hours after a transfer, worms are not necessarily fully adapted to a new salinity.

There is little difference in the appearance of these curves when compared with those obtained after a downward transfer over the same salinity range (Fig. 5a). Rates are slower and equilibrium with the new salinity takes longer in *N. limnicola* than in *N. succinea* or *N. vexillosa.*

3. Downward transfer out of the salinity range of osmotic conformity. *N. limnicola,* after adaptation to 49% sea water (272 mM Cl^-), was transferred to 5% sea water (27 mM Cl^-), well into the salinity range of hyperosmotic regulation (two experiments). The concentration gradient set up between internal and external fluids was 245 mM Cl^-, which would be reduced to about 100 mM Cl^- after full adaptation, due to the hyperosmotic body fluids in the new, lower salinity. The results (an average of 19 worms per point) are graphed in Figure 6a. As in the downward transfer entirely within the salinity range of osmotic conformity (Fig. 5a), there was an immediate decrease in the concentration of total body chloride, which reached a value not statistically different from that expected in worms adapted to 5% sea water (black arrow) by the eighth hour after transfer. Maximal water (weight) gain was by the eighth hour, followed by volume reduction. The net chloride loss had a maximum rate of $-39\ \mu M$ Cl^-/gm.-hr., in the first half-hour after transfer, and an average rate of $-15\ \mu M$ Cl^-/gm.-hr. for the entire first hour. This latter rate is somewhat higher than the maximum rate found in *N. succinea* for a less severe transfer from 50% to 23% sea water (initial gradient 159 mM Cl^-; maximum rate $-8\ \mu M$ Cl^-/gm.-hr.) (Oglesby, 1965b).

4. Upward transfer out of the salinity range of hyperosmotic regulation. N. limnicola, after adaptation to 4% sea water (24 mM Cl^-), was transferred to 51% sea water (283 mM Cl^-) (three experiments). Although the salinity transfer is the exact reverse of that just described, the initial concentration gradient set up between internal and external fluids is considerably less, about 115 mM Cl^-, due to the hyperosmotic body fluids of the worms in the old, lower salinity. The gradient would be reduced to zero after full adaptation to the new salinity. The results (an average of 28 worms per point) are graphed in Figure 6b. After the transfer there was a slow increase in the concentration of total body chloride, not reaching a value statistically the same as that for adapted worms (black arrow) for 24 hours. There was further change in concentration between 24 and 48 hours. There was an immediate loss of water, with a maximum delayed (as compared with Figs. 5b and 5a) to 12 hours after the transfer. This was followed by volume regulation, not yet complete after 48 hours. Net chloride gain was slow, with a maximum rate of $+6\ \mu M$ Cl^-/gm.-hr., in the second half hour after trans-

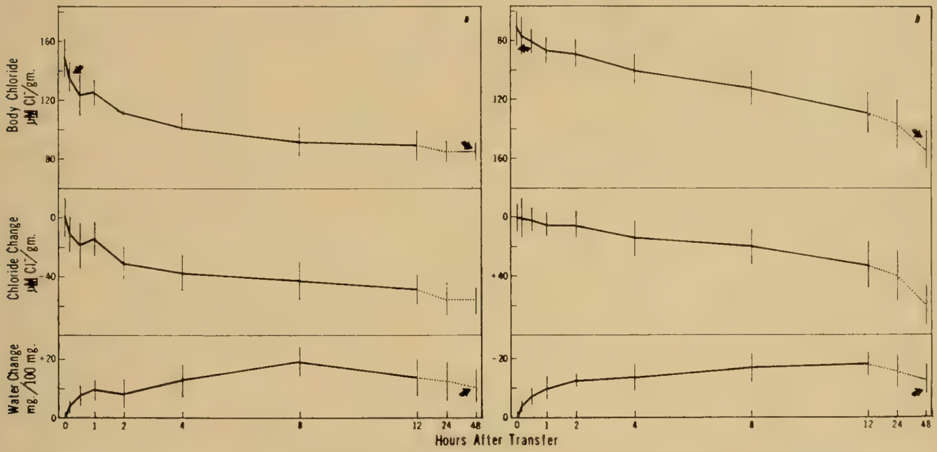

FIGURE 6. *Nereis limnicola* transferred into, and out of, salinity range of hyperosmotic regulation. For explanations, see Figure 5. (a) Transfer from 272 mM Cl^- to 27 mM Cl^- (N = 192). (b) Transfer from 24 mM Cl^- to 283 mM Cl^- (N = 276).

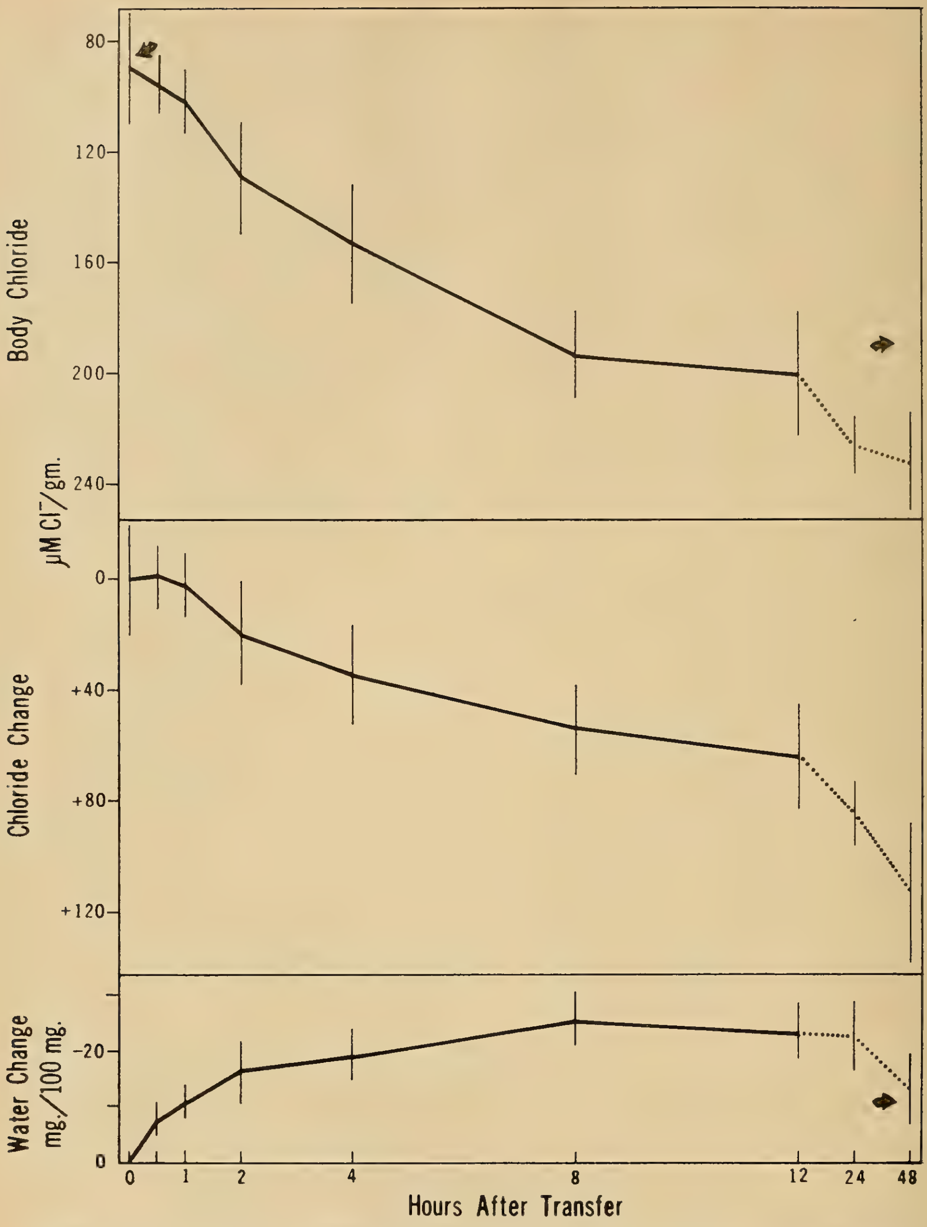

FIGURE 7. *Nereis limnicola* transferred out of salinity range of hyperosmotic regulation, from 17 m*M* Cl^- to 353 m*M* Cl^- (N = 132). For explanation, see Fig. 5.

fer, and an average rate over the entire first hour of +5 µ*M* Cl^-/gm.-hr. The rate subsequently declined. A higher rate of chloride gain was found in *N. succinea* after a less severe transfer from 22% to 50% sea water (initial gradient about 100 m*M* Cl^-; maximum rate +12µ*M* Cl^-/gm.-hr.) (Oglesby, 1965b).

It has been pointed out (R. I. Smith, pers. comm.) that the low rates of chloride and water movements found in these worms transferred from 4% to 51% sea water might be a consequence of the smaller initial concentration gradient (115 m*M* Cl^-) set up, as compared to the larger gradients set up in the other transfers with *N. limnicola* (221–245 m*M* Cl^-). To test this, *N. limnicola,* after adaptation to 3% sea water (17 m*M* Cl^-), was transferred to 63% sea water (353 m*M* Cl^-) (two experiments). The initial concentration gradient set up between internal and external fluids was about 213 m*M* Cl^-, which would be reduced to zero upon full adaptation to the new salinity. The results (an average of 15 worms per point) are graphed in Figure 7. The initial rates of chloride concentration change, water loss, and net chloride gain are all about the same as in the upward transfer from 4% to 51% sea water just described. Within the first hour, the maximal rate of net chloride gain was less than +3 μM Cl^-/gm.-hr., a value not distinguishable statistically from zero, and less than that found in the worms transferred from 4% to 51% sea water. In the second hour, the rate of chloride gain increased to +17 μM Cl^-/gm.-hr., but declined thereafter. Rates of water loss and of chloride concentration change also increased after the first hour, becoming more comparable to those found in transfers involving worms adapted to a salinity in the range of osmoconformity. Thus it appears that the low initial rates of chloride and water movements found in worms adapted to low salinities are a consequence of adaptation to low salinity, rather than to a reduced concentration gradient.

This series of experiments demonstrates that rates of chloride movements in *N. limnicola* (and also *N. succinea*: Oglesby, 1965b) are lower in worms adapted to a salinity in which they osmoregulate than it is after they have been adapted to a salinity to which they conform. Furthermore, rates of chloride movement, both gain and loss, are lower in *N. limnicola* than they are in *N. succinea* for similar transfers.

DISCUSSION

Hyperosmotic regulation in low salinities, coupled with osmotic conformity in higher salinities, seems to be the pattern found in osmoregulating nereid polychaetes. Such a pattern has now been demonstrated in five species of the Nereidae: *Nereis limnicola, N. succinea,* and *Laconereis culveri* (see introduction), *N. diversicolor* (Hohendorf, 1963; Smith, 1955), and, to a lesser extent, *Perinereis cultrifera* (Grube) (Jeuniaux, Duchâteau-Bosson, and Florkin, 1961; Panikkar, in Wells and Ledingham, 1940; Zenkevich, 1938a, 1938b). It is likely that another brackish-water nereid, *Tylorrhynchus heterochetus* (Quatrefages), is hyperosmotic in low salinities (Kamada and Yamamoto, 1931, as *Ceratocephale osawai* Izuka). Two other nereid species, *N. vexillosa* (Oglesby, 1965a) and *Nereis* (*Nereis*) *pelagica* Linnaeus (Schlieper, 1929), do not appear to osmoregulate.

Osmotic regulation has not been unequivocally demonstrated in any species of other polychaete families. In the Arenicolidae, both *Arenicola marina* (Linnaeus) (Ginetzinsky, 1959; Schlieper, 1929) and *Arenicolides branchialis* (Audouin and Milne Edwards) (Zenkevich, 1938b, as *Arenicola grubei* Claparède) are reported to be osmotic conformers down to the lower limits of their survival (30–50% sea water). In the Onuphidae, *Onuphis magna* (Andrews) is reported to have a high level of hyperosmotic regulation in salinities below about 60% sea water (Ebbs and Staiger, 1965), a level much higher than found in any nereid species. However, the measurements were few in number (1–2 worms per point) and were taken only 2–3 hours after transfer to lower salinities. Survival was extremely short in even moderately low salinities (fewer than 12 hours, apparently). Therefore, these cannot be considered as acclimated worms. *O. magna* appears to have very little tolerance for low salinities.

Krishnamoorthi (1963b) and Krishnamoorthi and Krishnaswamy (1965, 1966b, 1966c) provide considerable data on *Marphysa gravelyi* Southern, a brackish-water eunicid found naturally in waters no lower than about 13% sea water (Krishnamoorthi, 1963a), and which is not able to live in lower salinities in the laboratory (Krishnamoorthi and Krishnaswamy, 1966a). These authors conclude from their data that *M. gravelyi* not only is hyperosmotic in low salinities, but is also hypoosmotic in high salinities, an osmotic response not otherwise reported in any polychaete (see Oglesby, 1968). There were similar conclusions concerning regulation of the ions Na^+, K^+, and Cl^-. However, careful inspection of methods and data in these papers suggests that there may have been serious problems with analytical techniques, as well as with graphic presentation. When converted uniformly to millimolar units, their data are revealed to be incompatible. Any conclusions about ionic regulation, or hyper- and hypoosmotic regulation in *M. gravelyi* seem therefore to be inappropriate at the present time.

Studies by Smith (1963, 1964) have shown that rates of salt and water movements in nereid polychaetes are a function of body size. Larger worms have lower weight-specific rates than do smaller worms. Since it was not feasible in the present study to use worms of only a single size, worms were selected randomly in order to minimize the effects of different body sizes on average rates. However, the population from which they were taken did not have a normal distribution for size. Figure 8 shows a histogram of the weights (summed over intervals of 20 mg.) of 1474 *Nereis limnicola* weighed between spring, 1965, and spring, 1967. The stippled portion represents the weight distribution of the 972 worms used in the 11 osmotic stress experiments herein reported. These histograms are not truly representative of the size range of worms in the field. Small worms (less than 40 mg.) were usually overlooked or rejected during collection, and reproductive worms of any size were also rejected. For all 1474 worms, the median weight was 110 mg., and the average weight 130 mg. For the subsample of 972 worms used in the osmotic stress studies, the median weight was 90 mg., and the average, 110 mg. Average weights for worms in individual experiments did not vary more than about 10–20 mg. from the mean for all experiments. However, variability in worm sizes in the different experiments is undoubtedly an important factor contributing to variability in results.

The present experments on salt and water movements in *N. limnicola* when exposed to osmotic stress support previous evidence that *N. limnicola* has a body surface which is less permeable to salts (or has a reduced area of permeable body surface) than either *N. succinea* or *N. vexillosa* (Oglesby, 1965a; Smith, 1963). Smith (1963) has shown that weight-specific salt loss rate is lower in larger than in smaller individuals of several nereid species. Individuals of both *N. succinea* and *N. vexillosa* used by Oglesby (1965b) were considerably larger than the *N. limnicola* used in the present experiments. If there were no difference in the

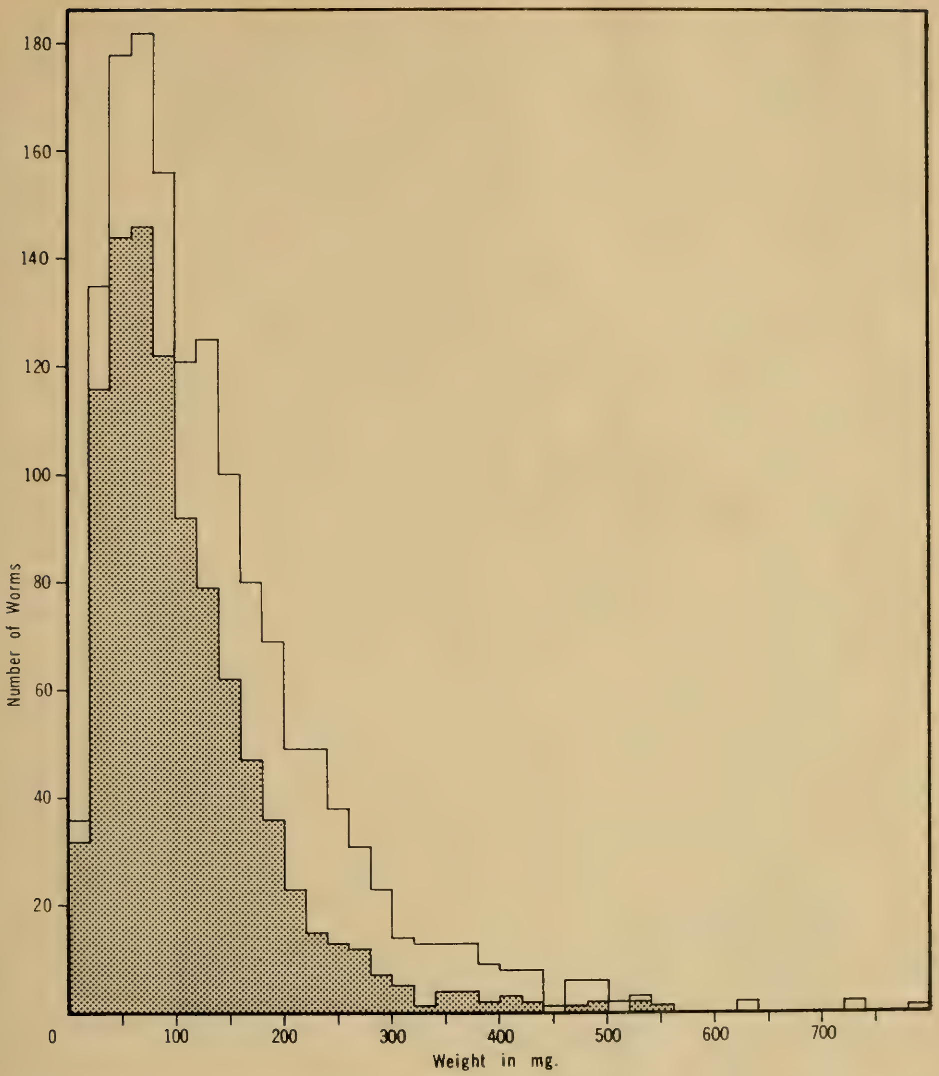

FIGURE 8. Frequency distribution of weights of *Nereis limnicola*. Entire histogram: weights of all 1474 worms weighed in 1965–67 ($\bar{X} = 130$ mg.; median, 110 mg.); stippled portion: weights of 972 worms used in osmotic stress experiments ($\bar{X} = 110$ mg.; median, 90 mg.).

total permeability of the body surface to salts in these three species, one would expect that the observed weight-specific rates of salt and chloride movements in the smaller *N. limnicola* would be higher than in the larger *N. succinea* and *N. vexillosa*. In fact, *N. limnicola* has lower rates of chloride and salt movements than either of the other species, after comparable transfers (Oglesby, 1965b, and present results; Smith, 1963).

On the basis of earlier results, Oglesby (1965b, 1968) hypothesized that the body surface of euryhaline nereid polychaetes, such as *N. limnicola* and *N. diversicolor,* is less permeable to salts when the worms are adapted to low salinities than it is when they are adapted to high salinities. These differences would have considerable significance in the physiological adaptation of the worms to life in low salinities and fresh water, in that diffusional salt loss would be much reduced. The results of the present experiments are completely consistent with this hypothesis.

However, one cannot attribute the site of the net salt movments measured in osmotic stress experiments solely to the body surface. Even if the urine were hypoosmotic to the body fluids (and there is no direct evidence that it is), there will be chloride loss in the urine. This urinary loss will result in the observed net gain of chloride after upward transfers being numerically less than the total uptake across the body surface alone; similarly, chloride loss in the urine will result in an observed net loss after downward transfers which is numerically greater than the total loss across the body surface alone. The magnitude of chloride loss in the urine is unknown, since there is no information on urine volumes and concentrations. Whether urine volume and concentration change in response to transfers or adaptation to different salinities is also unknown. It is not possible with osmotic stress experiments, which can measure only net movements of salts or water, to assess the relative importance of urinary and body surface routes. Hence it is not possible to conclude from the present observations, in which net chloride gain after upward transfers was lower than net chloride loss after downward transfers, that worms adapted to salinities in the osmoregulatory range must have a reduced permeability of the body surface to salts.

In the present experiments it was also observed that, after upward transfers, worms adapted to 3–5% sea water (Figs. 6b and 7) had considerably lower rates of chloride gain ($+3$–$+6$ μM Cl^-/gm.-hr.) than did worms adapted to 49% sea water (Fig. 5b; $+13$–$+15$ μM Cl^-/gm.-hr.). Since the effect of urinary chloride loss is the same in both instances (subtractive), this difference in net gain rates may be a consequence of a lower permeability to salts in the worms adapted to a salinity (3–5% sea water) in which they are osmoregulating.

The results of these osmotic stress experiments are therefore suggestive of a reduction of the permeability of the body surface to salts in worms adapted to low salinities; however, such experiments cannot provide a conclusive demonstration of this phenomenon. Problems in determining routes (urine, body surface) have already been mentioned. Additionally, electrical potential differences between the external medium and the body fluids may be of importance in ion regulation, especially in worms which are osmoregulating. There is no information available on the electrical potential across the body wall, and whether this varies with salinity of adaptation. Experiments with radioisotopes can determine total ion fluxes, and with this type of information and with electrical potential measurements, it may be possible to relate the results of the present osmotic stress experiments to hypotheses concerning reduction of permeability and active transport in low salinities. Preliminary work with radioisotope tracers (Oglesby, 1965b) did not support the hypothesis of active transport in low salinities, and indicated that permeability reduction might be important. Experiments with radioisotopes are continuing in this laboratory.

It has been hypothesized (Fretter, 1955; Jørgensen and Dales, 1957; Potts and

Parry, 1964) that hyperosmotic regulation by nereid polychaetes is accomplished in large part by the active uptake of salts from the medium. According to this hypothesis, worms in low salinities should be engaged in active transport. One might therefore expect that osmoregulating worms, when transferred to a higher salinity, would have, at least temporarily, this active transport of salts superimposed upon the passive diffusional influx of salt expected in the higher salinity. However, the low rate of net chloride gain found in worms initially adapted to very low salinities suggests that active transport may not be a significant adaptation in *N. limnicola.*

Smith (1963) found that *N. limnicola* from Lake Merced, when transferred from 50% sea water to distilled water, had a salt loss rate of about -20 μEq salt/gm.-hr., for a 100-mg. worm for the first half-hour after transfer. The corresponding rate found in the present study for worms with an average weight of about 110 mg. (Fig. 8) transferred from 49% to 5% sea water (Fig. 6a), was about -39 μM Cl^-/gm.-hr. The urinary contribution to chloride loss would probably be similar in both instances. The salt loss rate for Schooner Creek worms is about twice as high as that found in Lake Merced worms, for this particular transfer. Such a difference in results may be a consequence of the ability of these worms to reduce the permeability of their body surface to salts when in low salinities. If the amount of the reduction were proportional to the lowness of the salinity, one would expect a greater reduction in permeability in worms placed in distilled water than in those placed in 5% sea water. It is also possible that the Lake Merced population has a reduced permeability to salts in all salinities, as compared to the Schooner Creek worms.

Volume regulation, as it is usually determined by the criterion of tendency to return towards the initial volume (weight) after a transfer, is not much evident in the present results. But the maximum net water uptake or loss under the influence of the osmotic gradients set up in these experiments was not much greater than the value expected for worms fully adapted to the new salinities. Therefore, there was relatively little excess water gain or loss to be compensated for by volume regulation. These experiments indicate that this species has good control over its body volume even after a large transfer. This supports the observations of Oglesby (1965a) and Smith (1957, 1959, 1964) that *N. limnicola* is an excellent regulator of its body volume. In this respect *N. limnicola* is even better adapted for life in low salinities than is the closely related *N. diversicolor*. *N. diversicolor* gains considerably more water after transfers than does *N. limnicola* (*e.g.,* Beadle, 1931, 1937; Ellis, 1937; Zenkevich, 1938a; see Oglesby, 1968, for review).

There are few differences in osmoregulatory ability and other osmotic responses between the fresh-water population of *N. limnicola* in Lake Merced studied earlier (Oglesby, 1965a, 1965b; Smith, 1957, 1959, 1963, 1964), and the estuarine population from Schooner Creek (present report). The Lake Merced population may have: (1) a slightly higher level of hyperosmotic regulation; (2) a slightly lower critical low salinity; and (3) a somewhat lower permeability of the body surface to salts. These features may be important in the adaptation of *N. limnicola* to permanent life in fresh water. Differences in osmotic responses among the several fresh-water and estuarine populations of *N. limnicola* that have been studied so far are, however, slight when compared with the osmotic responses of less euryhaline species. Therefore, they may not signify any real physiological differences between populations within the single species.

The manner of reproduction in this species, self-fertilizing, viviparous hermaphroditism (Smith, 1950), should be an effective agent isolating different populations from reproductive contact with each other, especially since estuaries on the West Coast are usually separated from each other by long stretches of rocky or sandy coastline apparently not suitable for *N. limnicola.* In the absence of genetic exchange between populations in different habitats, one would expect different adaptive physiological responses to become stabilized. What is surprising about the ecologically distinct populations of *N. limnicola* is not that there are a few physiological differences, but that these differences are so small.

It is a pleasure to thank Dr. Ralph I. Smith, Dr. G. Frank Gwilliam, Prof. John Shaw, and my wife, Dr. Alice Shoemaker Oglesby, who have read the manuscript and made many helpful suggestions. I appreciate the technical assistance of Mr. Kermit C. Edwards and Mr. Lawrence V. Majovski. They, and Mr. Arthur Feinstein, helped me collect worms in fair weather and foul, even when they didn't have to. This study was supported by a research grant from Reed College, and by National Science Foundation Grant GB-4429.

SUMMARY

1. The ecology of an estuarine population of the euryhaline nereid polychaete *Nereis limnicola* is briefly described. Seasonally this population is exposed to salinities varying from nearly fresh water to at least 85% sea water. Tidal variation is minimal.

2. The pattern of chloride regulation in the body fluids is similar to that described for four other nereid species: osmotic and ionic conformity at salinities above 30% sea water; and hyperosmotic regulation in lower salinities, down to a critical low salinity at which hyperosmotic regulation begins to break down (about 1–3% sea water). There is little difference in this pattern between fresh-water and estuarine populations of *N. limnicola.*

3. After a transfer from one salinity to another, both salinities within the range of osmotic conformity, there is an immediate change in the total body chloride of the worms, due both to a movement of water and to a movement of chloride, in the directions of the imposed concentration gradients. When worms are adapted to low salinities in the range of hyperosmotic regulation, rates of chloride movements are much reduced after a transfer.

4. Complete adaptation to a new salinity after a transfer may take more than two days.

5. Rates of chloride and water movement are lower in *N. limnicola* than in several less euryhaline species.

6. The results suggest that *N. limnicola* is less permeable to salts, and perhaps also to water, than the less euryhaline species, and that permeability may be further reduced after adaptation to low salinities. These features are considered to be of significance in the physiological adaptation of *N. limnicola* to life in low salinities and fresh water, in that diffusional salt loss is much reduced.

7. The results do not support the hypothesis that active transport of salts from the medium across the body surface is important in the maintenance of hyperosmotic body fluids.

8. The fresh-water population of *N. limnicola* previously studied may have: (a) A slightly higher level of hyperosmotic regulation. (b) A slightly lower critical low salinity. (c) A somewhat lower permeability of the body surface to salts. However, differences in osmotic responses among the several brackish- and fresh-water populations of *N. limnicola* are slight when compared with the osmotic responses of less euryhaline species.

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BANDING PATTERNS OF HALIOTIS RUFESCENS AS INDICATORS OF BOTANICAL AND ANIMAL SUCCESSION [1]

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Although it is reasonably well established that tree rings can be used as indicators of environmental fluctuations (*e.g.,* Fritts, 1966), there have apparently been few attempts to develop a similar technique for the marine environment. Growth rings are seen in the stipes of the brown alga *Laminaria hyperborea* (Kain, 1963) and represent up to four year's growth. Because of the short time interval involved in their formation they would consequently be of little use as indicators. Since some molluscs live for more than 50 years (Comfort, 1957), it would seem that the rings found in their shells would be useful indicators of long-term environmental fluctuations. For the the establishment of such a system we would need to know the time interval involved in the formation of the rings and the environmental fluctuations that cause variations in the rings. If the relationship between environmental variation and the growth rings can be established the investigator may be able to use the information contained in the rings to make statements about the environment from which the shells came.

Moore (1936) related shell coloration of *Purpura lapillus* to fluctuations in abundance of this predatory gastropod's main food source, *Mytilus.* He also attempted to use the shell color patterns to explain distributional patterns which are characteristic of the early phases of the life history.

Recently Forster (1967) studied the growth of *Haliotis tuberculata* and discovered that this species normally forms an annual ring in the shell. *H. tuberculata* forms shell at the rate of about 15 mm. per year. Forster also discusses the value of annual rings in computing growth rates for studies of fishery yield.

Ino (1949, 1953), in a series of feeding experiments, found that shell coloration in *Turbo cornutus* and *Haliotis discus* could be explained by changes in the types of algae that the animals fed upon, but made no attempt to utilize this coloration to refer back to the environment from which the animals came. Leighton (1959, 1961, 1963) has studied the effects of changes in the types of algae in the diet of *Haliotis rufescens* and *H. cracherodii* upon the shell color. He suggests that color sequences in the shells of this animal (*H. rufescens*) could be used as a key to botanical succession in the home area of the respective animals. His results indicate that red algae cause the mantle of *H. rufescens* to secrete a red shell color while brown and green algae result in white or very light tints of green or blue. Commercial abalone cultivation attempts were made in Morro Bay and Pigeon Point, California. It was found that when abalones are fed diatoms the shell that

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is secreted is a vivid turquoise. Leighton (personal communication) agrees with this finding.

The brown alga *Nereocystis luetkeana* first becomes available in April when the plants are about one foot in height (McLean, 1962). At this time it probably does not make up a very large proportion of the abalone's diet. Figure 1 shows that it continues to grow and reaches maximum density in late summer or early fall. Throughout the fall and winter, storms tear the stipes loose and cast them ashore. Until June or July, the stipes are almost completely free of red algal epiphytes (Markham, 1963). These epiphytes reach their maximum in September and October and then are lost when the host stipes are defoliated. In the Morro Bay area, commercial abalone fishermen also contribute to *Nereocystis* mortality by cutting the floating stipes and causing them to sink to the bottom where they are eaten by abalones and other animals.

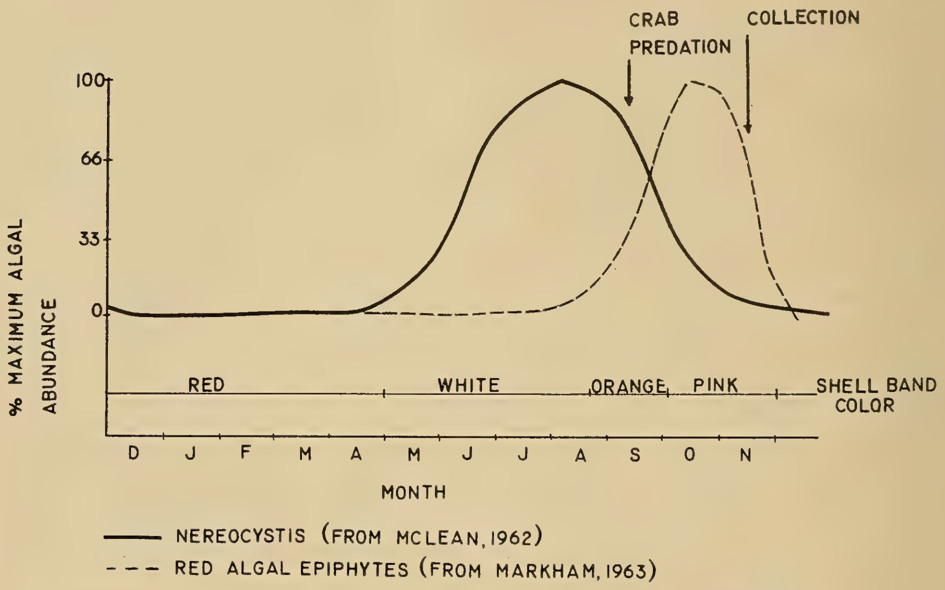


FIGURE 1. Seasonal succession of *Nereocystis luetkeana* and its relation to shell color in *Haliotis rufescens*.

Leighton (1966) and Sakai (1962) have demonstrated that abalones exhibit preferences between different types of algae. Generally they appear to prefer brown algae. In this paper, it is assumed that *H. rufescens* exercises this preference for *Nereocystis*. Abalones are frequently fed this alga at the commercial abalone culture facilities in California. *Nereocystis* grows abundantly in Morro Bay.

If we can assume that *H. rufescens* exercises a preference for *Nereocystis*, the shell of the abalone should be white for the period between April when *Nereocystis* first becomes available, and June or July, when the red algal epiphytes first begin to appear. From July until December the abalone will secrete a shell that is increasingly reddened by the increasing proportion of red-algal epiphytes that are ingested with the brown. The trend from orange to pink should represent this period. The time interval from December to April should be represented by a red shell color because the abalone will be forced to shift over to an alternate diet of red algae when the *Nereocystis* is defoliated by storm action. Neushul (1965) demonstrated that there is an abundance of red algae available at this season (Fig. 1).

From 1957 to 1960 there was a period of unusually warm water (Radovich, 1961) which killed off most of the *Nereocystis* in the vicinity of Morro Bay (Cox, 1962). Since abalones live for up to 13 years (Comfort, 1957), those abalones that were alive in this period should have a broad band of red representing 4 year's diet of red algae. If the yearly diet cycle is true, then there will be six repetitions of it in these shells. Outside of the broad red band, the most recent band should be pink because the shells were collected in November. If the seasonal cycle of *Nereocystis* is represented in the shells, then the shell will serve as a record of the history of algal abundance in the abalones' home area because Cox (1962) has shown that the red abalone does not move from that area.

In addition, the shells preserve a record of the predatory actions of crabs (probably of the genus *Cancer*) who break the abalones' shells. Commercial abalone fishermen from Morro Bay report that there is a late summer invasion of rock crabs who break the abalones' shells. The crabs in turn are reportedly preyed upon by octopuses who come in when the water cools down in October or November. Most of this crab damage should be recorded in the orange band.

The purpose of this paper is to test the hypothesis that the banding patterns of *H. rufescens* can be related to the yearly cycle of *Nereocystis*. The evidence to be considered will be (1) the order of the colors in a sequence that corresponds to the cycle; (2) the frequency of the various colors; (3) the color of the most recent band; (4) the color of the bands damaged by crabs; and (5) the number of repetitions (if any) of the color sequences in those shells having the wide red band resulting from the *Nereocystis* die-back caused by warm water.

Materials and Methods

Seventy-five shells were obtained from a commercial fisherman who collected them in November, 1966. These shells were all between 197 mm. and 235 mm. since the state of California places a $7\frac{3}{4}$-inch minimum size restriction upon the commercial abalone fisherman. The shells were returned to the lab and cleaned with a wire brush and hydrochloric acid to make the colored bands visible. The color sequence was recorded from the outer edge of the shell inward to the parts of the shell secreted by the young animal. The length, width, and weight of each shell, along with the width of each individual band and the total number of bands for each shell, were also recorded. The color sequences from each shell were analyzed with a computer program that did the following operations: (1) The frequency with which each individual color appeared was counted and recorded; (2) every possible four-color sequence was counted and recorded.

Four color sequences were chosen for convenience. In this analysis one color band cannot be followed by a band of the same color. This is based on the assumption that a single band does not represent more than one year's growth.

Cox (1962) has found up to 48 mm. growth in tagged abalones which were recaptured in one year. This figure is roughly the same size as the largest bands found in this study.

RESULTS

Forty-five of the 75 shells exhibited some degree of banding. The total number of bands for each individual ranged from 5 to 60 with a mean of 23. There was a total of 1034 bands. Five relatively distinct colors were found in the shells of *Haliotis rufescens.* For purposes of presentation these colors are roughly comparable to the colors 4-4 (red), 5-c-2 (pink), 3 (orange), 9-d or 10-d (white), and 9-1 (blue) in Cheskin (1954). Table I shows the frequency of occurrence for each of the five colors. The expected value was obtained by multiplying the proportion of a year that the condition exists which will allow that shell color to be secreted by the abalone times the total number of bands. Since *Nereocystis* and its epiphytes are rare for about the first month of their growing season, red shell could be secreted throughout 5 months of the year, pink during 2 months, orange during about one month, and white during 4 months. The single color occurrences as shown in Table I did not differ significantly at the 0.02 level from the expected frequency. The blue bands have been deleted from this total since they are restricted in their occurrence.

TABLE I

Single color occurrences

Color	Observed	Expected*
Red	405	387
Pink	130	156
Orange	94	77
White	301	310
Total	930	930 $\chi^2 = 8.20$, $.05 > P > .02$

* Based on the proportion of the year that the condition for that color to be secreted exists multiplied times the total. The total for blue was deleted because it is restricted in its occurrence.

The 1,034 single color bands could be classed into 899 four-color sequences. Since there are 320 four-color sequences possible when one utilizes five colors with no color permitted to follow itself, the expected frequency for an even distribution was 2.81 or 0.31%. The 20 most frequent combinations occurred in 580 of the possible 899 cases, or 62.6% of the time. The remainder were divided up more or less evenly among the other 300 possibilities. The 20 most frequent four-color sequences are listed in Table II. Of the 20 most frequent, only three involved red followed by anything but white or blue. Each of these three was a red-to-pink alternation that probably means that the abalone ate mostly red algae in those years because the *Nereocystis* was scarce.

The number of pink bands which were on the outside edge of the shell was significantly higher than would be expected on the basis of the single occurrence

TABLE II

The twenty most frequently observed sequences of four colors

Sequence	Observed	% of Total
RWRW	104	11.5
WRWR	94	10.5
RBRB	60	6.7
BRBR	48	5.3
WRWP	27	3.0
RWPW	26	2.9
WORW	23	2.6
RWOR	21	2.3
WPWR	21	2.3
ORWR	20	2.2
WPWP	20	2.2
RWRB	17	1.9
WRBR	15	1.7
PRWR	14	1.6
WRWO	13	1.4
RPRW	12	1.3
RWPR	12	1.3
PRPR	12	1.3
PRWP	11	1.2
ORWO	10	1.1
Total	580	62.2%

R = red, P = pink, O = orange, W = white, B = blue.

of the colors. This finding is shown in Table III and is expected since the shells were collected in November.

Eighty-nine per cent of the shells had breakage in one or more of the bands. In all there were 95 damaged bands. The number of orange bands shown in Table IV was significantly higher than would be expected on the basis of the single color occurrences.

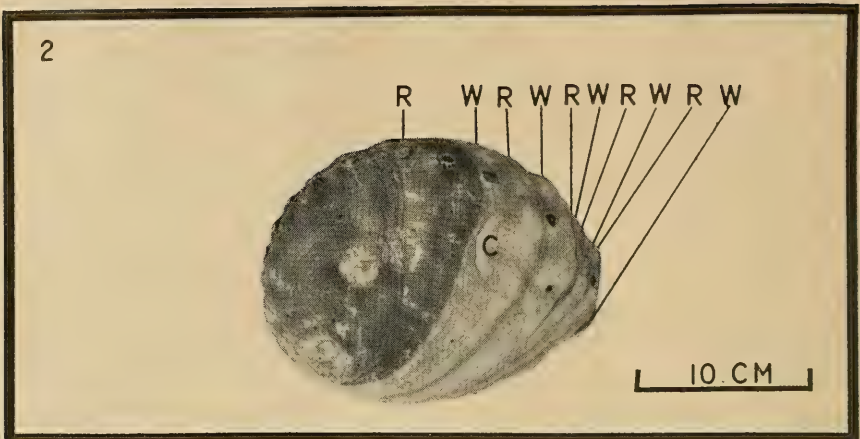
Five of the shells had an area of solid red, which obviously represented several years' growth, followed by a typical series of bands. Figure 2 shows one of these

TABLE III

Color of the most recent band at the edge of the shell

Color	Observed	Expected*
Red	15	19
Pink	14	6
Orange	5	5
White	11	15
Blue	0	0
Total	45	45 $\chi^2 = 11.44$, $.001 < P < .01$

* Based on single color occurrences.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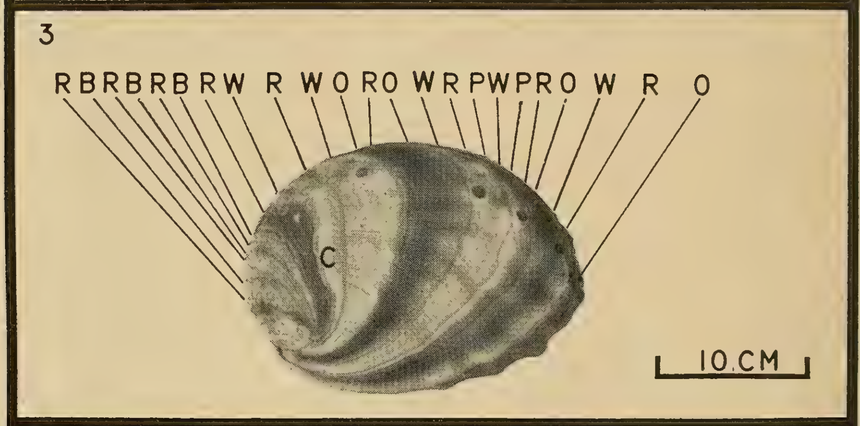
C
10 CM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R B R B R B R W R W O R O W R P W P R O W R O
C
10 CM

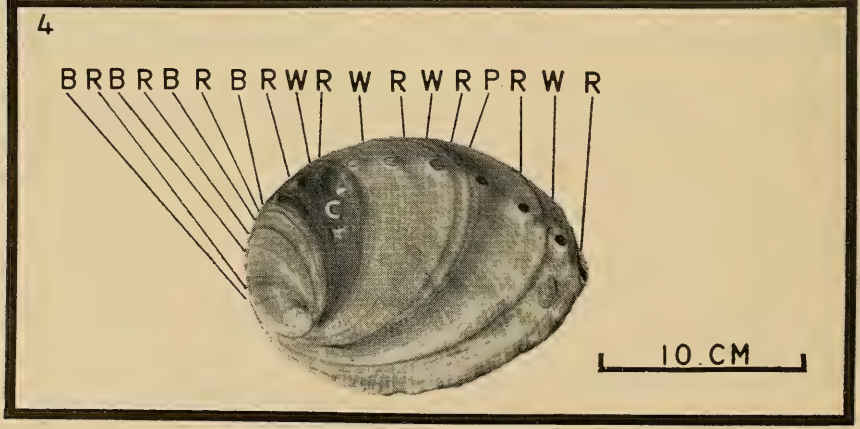
4
B R B R B R B R W R W R W R P R W R
10 CM

FIGURE 2. An abalone shell showing a large area of red followed by a series of alternations of white to red. The large red area resulted from a period of warm water from 1957 to 1960 which killed the *Nereocystis* in the abalone's home area.

TABLE IV

Color of bands damaged by crabs

Color	Observed	Expected*
Red	44	37
Pink	12	12
Orange	18	9
White	18	28
Blue	3	9
Total	95	95 $\chi^2 = 19.76$, $P < .001$

* Based on single color occurrences.

shells. In each of these shells there were 6 red bands between the solid red area and the last band which represented the time of collection.

The average distance from one red band to (but not including) the next one was 17.13 mm. This represents the average growth for a single year. This figure agrees remarkably well with Forster's (1967) figure of 15 mm. yearly growth for *H. tuberculata.*

DISCUSSION AND SUMMARY

The striking banding patterns in the shells of *Haliotis rufescens* almost certainly represent changes in diet and are probably not of selective importance since they are completely masked by the ostracum and a growth of epiphytic algae and animals. This masking also eliminates the possibility of selectively collecting banded shells. *Nereocystis* is not available or not preferred by all of the animals, as evidenced by the 30 solid red shells in our sample. The alternation between pink and red in several of the shells also indicates that the abalones are sometimes unable to obtain *Nereocystis* in sufficient quantity to make the shell white.

Until more complete feeding experiments have been completed, we must assume that the colors orange and pink represent an increasing proportion of red algae in the diet. Red algae discolor the white caused by the abalone's preference for brown algae as demonstrated by Leighton (1966).

The 20 most frequent four-color sequences shown in Table II show a definite trend for either a red-to-white alternation or a series involving white, orange, or pink, and red. There is a very definite absence of series with red followed by pink or orange, then white. These findings, along with the high number of pink first bands and the high number of orange bands that had been damaged by crabs, support the idea that the yearly cycle of *Nereocystis* reported by McLean (1962)

FIGURE 3. An abalone shell from Morro Bay, an area where the predominant brown alga is *Nereocystis.* Note the frequent occurrence of red and pink bands indicating periods when the *Nereocystis* is covered with red algal epiphytes.

FIGURE 4. An abalone shell from Goleta, an area where the predominant brown alga is the perennial kelp *Macrocystis.* Note the wider white bands, narrow red bands and relative absence of pink and orange colored bands. r = red, w = white, p = pink, o = orange, b = blue, c = damage caused by predatory rock crabs.

and the epiphyte cycle reported by Markham (1963) are recorded in the shells of *H. rufescens.* Additional evidence can be found in the shells of the abalones that had been alive in the warm-water period (Radovich, 1961) which killed the *Nereocystis* in the Morro Bay area. In these shells the period of warm water is recorded as a wide band of red. The interval since that period is recorded by a series of six repetitions of the basic red-to-white alternation. This is shown in Figure 2. The small number of these shells in the population is probably due to the fact that these abalones are approaching the upper limits of the age distribution. The smaller abalones would have eaten diatoms when available. Only those animals that were greater than about 80 mm. at the time when *Nereocystis* was absent would have this wide band in the shell.

Further evidence for the preservation of the algal cycle arises from a comparison of shells from an area where *Macrocystis* is the predominant brown alga with those from Morro Bay. Figure 3 shows a typical shell from Morro Bay where *Nereocystis* is the predominant brown alga. There are frequent occurrences of intermediate colors between a white-to-red succession. The shell in Figure 4 was collected from south of Point Conception in an area where *Macrocystis* is the predominant brown alga. Unlike *Nereocystis, Macrocystis* is a perennial (Fritsch, 1959) and is available to the abalones throughout most of the year. *Macrocystis* is also relatively free of red algal epiphytes. There is almost a complete absence of intermediate colors on this shell as would be expected. The white bands are also very much wider than the red bands since the abalones can obtain *Macrocystis* throughout most of the year.

In this preliminary study a technique has been presented which, if developed and refined, could allow sublittoral ecologists to overcome some of the limitations inherent in underwater studies. It would make it possible to make relatively specific statements about study areas for a long time interval. It can also be used to show quantitative differences of seasonally fluctuating factors in the abalone's environment.

In order to refine this technique, the feeding experiments of Leighton, Ino and others need to be enlarged and expanded to the other species of *Haliotis* which exhibit shell banding. *Haliotis rufescens* provides a good basis for a dating system because the variations of color in the bands allow the investigator to obtain qualitative information. The yearly color sequences provide a method of dating, while band widths give an indication of growth rate relative to season and diet. Replicates of the current study should be undertaken for shells from regions where the recent history has been documented by other sublittoral studies. The relation between banding and environment can be more clearly established by long-term mark and recapture studies, gut content analysis and direct observation by diving.

Eventually, it might be possible to predict yield from fisheries on the basis of findings from "mollusc ring" studies, much as Ottestad (1960) has attempted with pine tree rings and the spawning cod fishery in Norway.

I would like to thank Doctors Joseph Connell, Adrian Wenner, and Michael Neushul for their advice and reading of the manuscript; Hugh Schellenberger for help in programming; Chuck Sitz for furnishing the shells; Carol Yant for typing; and my wife, Susan, for her aid in several aspects of the study.

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FEEDING HABITS, BEHAVIOR AND HOST SPECIFICITY OF CAPRELLA GRAHAMI, AN AMPHIPOD COMMENSAL WITH THE STARFISH ASTERIAS FORBESI[1]

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Caprella grahami Wigley and Shave, 1966, has been described from the coastal waters of southern New England, where it was found living on the starfish *Asteris forbesi* (Desor) and *A. vulgaris* Verrill. The present paper deals with aspects of the biology of this caprellid and with the relationships existing between it and its starfish host.

While *C. grahami* has been only recently discovered, there is a well known association involving European starfish and the caprellid *Pariambus typicus* (Krøyer). This species occurs most commonly on *Asterias rubens* L., but has also been reported from *Crossaster papposus* (L.) and occasionally as free-living (Cuénot, 1912). *C. papposus* commonly feeds on *A. rubens* (Mortensen, 1927), and this fact probably explains the occurrence of the caprellid on starfish belonging to two different orders. *P. typicus* is a small species, with males attaining a length of only 5–6.5 mm. (Chevreux and Fage, 1925). It is semi-transparent, does not excite host pedicellariae and occurs on the aboral surface or in the ambulacral grooves of the starfish (Cuénot, 1912).

I am indebted to J. C. McCain for kindly loaning me his manuscript bibliography of the caprellids and to M. R. Carriker, J. C. McCain and R. L. Wigley for critical reading of the manuscript. The photographs in Figure 1 were taken by J. L. Simon. This study was supported by a Faculty Fellowship from Ohio Wesleyan University.

OBSERVATIONS AND CONCLUSIONS

Caprella grahami was found to be extremely common on individuals of *Asterias forbesi* occurring at the rock jetty at the eastern entrance to the Cape Cod Canal, near Sandwich, Massachusetts. Specimens from this locality were collected from October to December, 1966, and brought to the Marine Biological Laboratory for study. They were maintained on their hosts in tanks of running sea water and survived well under laboratory conditions.

C. grahami is a relatively large caprellid, with males ranging up to 18 mm. in length (Wigley and Shave, 1966). The bodies of most specimens examined were transparent and only faintly colored. Some, mainly females, had diffuse cream-colored bands across the segments, while a few had the yellow-orange cast or the patches of red noticed by Wigley and Shave (1966).

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[1] Contribution No. 109 from the Systematics-Ecology Program.

Caprellids were found on all parts of the starfish. When disturbed they usually moved rapidly over the body in characteristic measuring-worm fashion, alternately attaching with the anterior appendages and with the last three pairs of legs. The dactyls of the appendages often dug into the soft epidermis of the host, but were never seen to pierce through it. When two starfish touched, the caprellids readily crossed from one to the other. Except for this, they were never observed to leave their host, nor were they ever seen in the debris around the starfish.

When a starfish was examined under 7 × magnification, the caprellids on the aboral surface could be seen quite easily against the dark color of the host. Here they are found crawling over the body or with the last three pairs of appendages clamped to the starfish and the front of the body extending above the host or laterally away from it (Fig. 1). A common position, especially among females, is to be pressed tightly against the body of the host and lying between or curling around its spines. White-banded individuals in this position are protectively colored. Not only do the alternate white and transparent bands break up the outline of the caprellid, but they also blend against the dark integument of the host with its scattered white spines. Sometimes specimens are found in the spine-enclosed vertical spaces formed between adjacent arms of the starfish and the central disc. Here they are completely out of sight and can only be discovered by passing a probe down between the spines. The first clue to the presence of caprellids on the light-colored oral surface of the starfish is often a movement of the reddish tips of the first antennae or sight of the dark line of gut contents. Individuals were commonly found crawling between the tube feet, moving around among the dermal papulae of the oral surface or lying in the grooves formed between the papulae and the adambulacral spines. They were sometimes found around the mouth, but were not seen to enter it. On several occasions a caprellid which

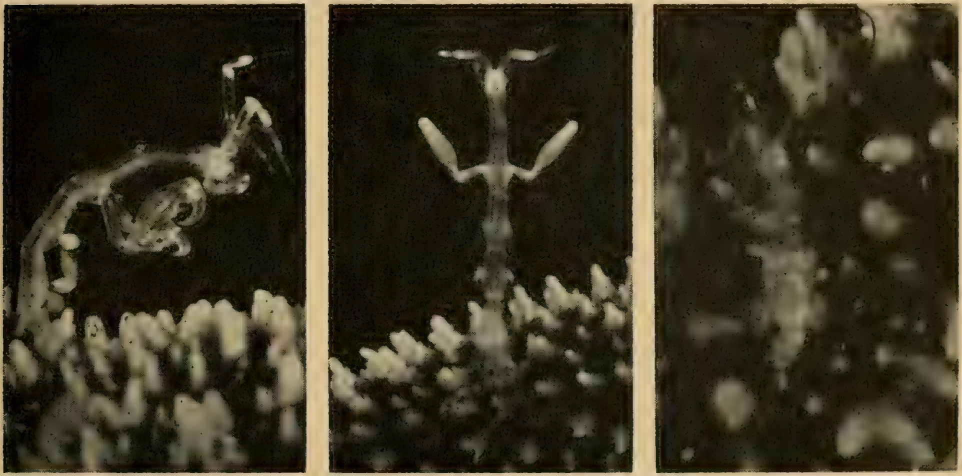

FIGURE 1. *Caprella grahami* in typical positions on *Asterias forbesi*. Left, male arched above the starfish. Center, male extending away from the host. Right, banded female pressed against the aboral surface. The body length of the specimens was approximately 8, 9 and 6 mm.

had been recently placed on a starfish could not be located despite careful searching (see experiments 3, 4 and 9 in Table I). Such specimens were generally found to have reappeared a short time later and had evidently been hidden out of sight or were motionless and thus had escaped detection.

Of 29 *Asterias forbesi,* ranging in diameter from 5 to 10 cm. which were collected individually on October 27, all but one bore caprellids, with the average incidence being 8.0 per starfish (Fig. 2). There was wide variation in the number of caprellids found, although the smaller starfish generally bore fewer than did the large ones. The highest incidence recorded was 40 specimens on a starfish 12 cm. in diameter, collected on November 22, from a part of the jetty that contained no other starfish. Both the first collection of starfish on October 13 and the last one, on December 10, as well as those made at times in between, contained caprellids of all sizes, including small juveniles and ovigerous females.

TABLE I

Host preference of Caprella grahami.
Each experiment involved an individual of A. forbesi and one of A. vulgaris placed in a bowl of sea water of sufficient size to allow freedom of movement yet insure periodic contact between starfish.

		Experiment number										Total
		1	2	3	4	5	6	7	8	9	10	
Approximate diameter of starfish in centimeters		10	12	9	10	9	11	9	3	20	5	
Location of caprellids	*A. forbesi*	0	5	0	5	0	5	0	5	0	5	25
at start of experiment	*A. vulgaris*	5	0	5	0	5	0	5	0	5	0	25
Location of caprellids	*A. forbesi*	5	5	4	4	4	5	3	4	2	5	41
after 3 hours	*A. vulgaris*	0	0	0	0	1	0	2	1	2	0	6
Specimens crossing from	*A. vulgaris* to *A. forbesi*	5	—	4	—	4	—	3	—	2	—	18
Specimens crossing from	*A. forbesi* to *A. vulgaris*	—	0	—	0	—	0	—	1	—	0	1

Feeding habits

Caprella grahami appears to feed largely on material found on the surface of the starfish. Freshly collected specimens generally have dark gut contents containing silt grains, amorphous material and small numbers of broken spicules and diatoms. Specimens maintained in the laboratory usually had brownish-green guts containing silt, amorphous material and diatoms. This material was identical to that settling out of the water system into the bottom of containers and presumably also onto the starfish. Carmine particles placed on the starfish were readily eaten by the caprellids, either as free particles or when coalesced by host secretions. Caprellids were seen to groom the spines and dermal papulae of the aboral surface and they doubtless pick up particles being carried up them by the distally beating cilia (Budington, 1942). Another likely source of detrital material for *C. grahami* is that picked up by the adhesive secretions of the host's tube feet. When starfish walked over carmine particles adhering to the bottom of a dish of sea water, they picked these particles up on their tube feet and shed them in strings of secreted material, which were then eaten by the commensals.

Durham (1888) described how dye particles injected into *Asterias rubens* were engulfed by ameboid cells and transported through the walls of the papulae where the cells disintegrated, releasing the dye particles. Starfish kept in the laboratory released a brown slime from the surface, which he felt was produced by a similar migration of ameboid cells through the papulae. He noticed incidentally that specimens of the caprellid *Pariambus typicus* ate both the ejected dye particles and the brown slime. I obtained identical results with *A. forbesi* and *C. grahami*. However, all the freshly collected caprellids that were examined had detrital material in their guts and thus I have no evidence that these host-produced substances are a major food for *C. grahami* under natural conditions.

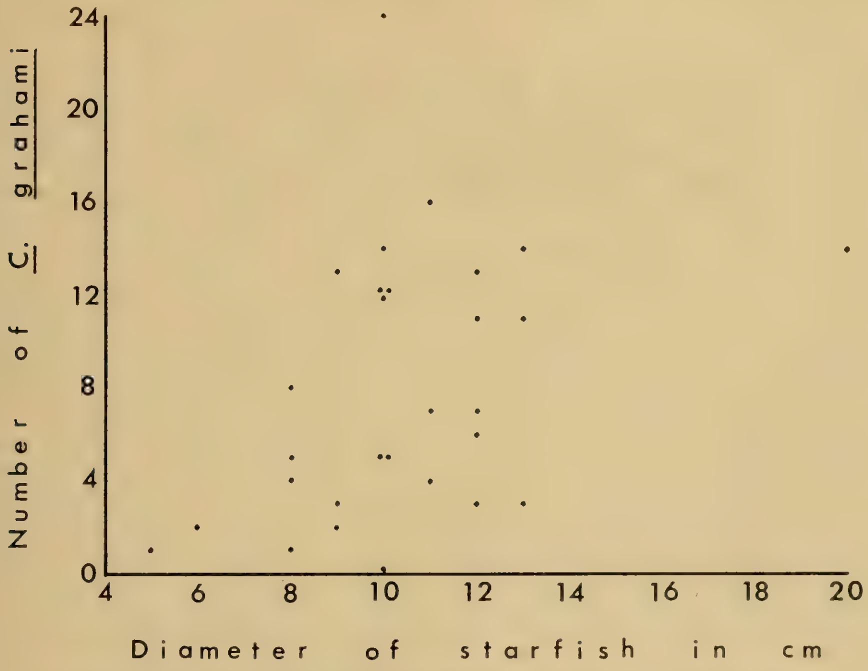

FIGURE 2. Incidence of *Caprella grahami* on 29 individuals of *Asterias forbesi* collected October 27, 1966.

Starfish were examined while feeding on mussels and there was no concentration of caprellids around the mouth and thus no evidence that the caprellids utilize host food.

Specimens of *C. grahami* seen extending away from the host certainly look as if they are waiting for food to come by. This type of posture followed by capture of organisms has been described for *Caprella dentata* by Wetzel (1932), and for

California caprellids by MacGinitie and MacGinitie (1949). However, animal remains were never seen in the guts of freshly collected *C. grahami,* nor would laboratory specimens capture *Artemia* nauplii.

It is not particularly surprising that *C. grahami* feeds mainly on detritus, as several species of caprellids remove sessile micro-organisms (mainly diatoms) from algae and hydroids (Wetzel, 1932; Costa, 1960; Saunders, 1966) and the feeding motions involved in collecting the two types of food would seem to be quite similar. Furthermore, in examining the gut contents of three caprellid species, Saunders (1966) found considerable unidentified material that may have been detritus.

Host preference

Both *A. forbesi* and *A. vulgaris* were common on the rocks at Sandwich Jetty. Wigley and Shave (1966) reported *C. grahami* on both species and I found it on specimens of *A. vulgaris* which had been brought back in a bucket also containing *A. forbesi.* Of eleven *A. vulgaris* collected individually on October 27, and brought back to the laboratory for examination, however, not one contained a single caprellid, while as shown in Figure 1, specimens of *A. forbesi* contained many. The experiments listed in Table I further demonstrate that *C. grahami* strongly prefers *A. forbesi* when given a choice of hosts.

To determine whether or not *C. grahami* can survive on *A. vulgaris,* seven individuals were placed on each of five *A. forbesi* and five *A. vulgaris* maintained in individual dishes and supplied with running sea water. The caprellids were counted every third day and the experiment concluded after 15 days. There was considerable mortality in both groups, probably due, at least in part, to the handling of specimens with forceps that was required for accurate counting. However, the mortality was markedly greater in the specimens on *A. vulgaris,* where only one of the original 35 caprellids survived for 15 days, as contrasted with 11 of 35 surviving for this period on *A. forbesi.*

Individuals of *C. grahami* kept on *A. vulgaris* generally contained material in the gut and appeared to differ from those on *A. forbesi* only in occurring less often on the oral surface of the host and in having the hind legs more often entangled in a mass of sticky material that was pulled along behind them.

Reaction of host

Jennings (1907) described in detail how the pedicellariae of the asteriid starfish *Astrometis sertulifera* (Xantus) grasp and hold amphipods and small crabs until they are eventually eaten. Numerous free-living caprellids, *C. geometrica* Say, were placed on specimens of *A. forbesi.* Most were reluctant to settle on the starfish and swam away from it. Those that did move over it were often held by the pedicellariae, but were usually able to pull loose. *C. grahami,* on the other hand, moves freely over the starfish and was seldom even briefly detained by the pedicellariae. In this regard it is interesting to note that the pedicellariae of *A. forbesi* are quite effective in holding small objects such as the hairs of a brush, and that the last three pairs of legs of *C. grahami,* which are in continual contact with the starfish, are practically devoid of spines and setae. *C. grahami* differs markedly in this respect from most caprellid species, including *Pariambus typicus* which occurs on *Asterias rubens* and has numerous setae on the last pairs of legs.

Discussion

Structural adaptation, as evidenced by the lack of spines and setae on the last three pairs of legs, the apparent host specificity and the reluctance of the animal to leave its host, all suggest that *Caprella grahami* is an obligate commensal. The fact that individuals of *Asterias forbesi* often collect together in favorable habitats, makes this species a most suitable host for *C. grahami*. Transfer of caprellids from host to host can readily occur despite the fact that *C. grahami* lacks any type of pelagic larval stage and that it apparently will not move from a host onto the substratum.

Summary

1. *Caprella grahami* was abundant on individuals of *Asterias forbesi* collected at Sandwich, Massachusetts. Commensals were found on all parts of the starfish and seemed to feed mainly on detritus that settled on the starfish or was caught up in host secretions.

2. Field collections and laboratory experiments indicate that *C. grahami* strongly prefers *A. forbesi* as a host over the sympatric *A. vulgaris*.

3. *C. grahami* is unusual among caprellids in lacking spines on the last three pairs of legs and is very rarely detained by host pedicellariae.

4. *C. grahami* crosses readily from one starfish to another, but was never found away from a starfish, and is considered to be an obligate commensal.

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NATURAL AND SYNTHETIC MATERIALS WITH INSECT HORMONE ACTIVITY. 3. JUVENILE HORMONE ACTIVITY OF DERIVATIVES OF p-(1,5-DIMETHYL-HEXYL) BENZOIC ACID

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Substances with high juvenile hormone activity have been found in the wood of certain evergreen trees, especially in the balsam fir *Abies balsamea* (Sláma and Williams, 1965, 1966). The active components were subsequently isolated and identified as juvabione (Bowers *et al.*, 1966) and dehydrojuvabione (Černý *et al.*, 1967). These terpenoid compounds, derived from the already known todomatuic acid, show juvenile hormone effects on insects of the family *Pyrrhocoridae*.

Thus far, nearly all active juvenile hormone materials have been aliphatic or alicyclic esters. We have now prepared the synthetic analogues of juvabione and dehydrojuvabione in which the alicyclic ring was replaced by an aromatic ring. Some of the compounds show surprising increases of juvenile hormone activity, while retaining their specific effects on insects of the family Pyrrhocoridae. The present paper describes certain relationships between biological activity and the chemical constitution of these new compounds.

Materials and Methods

For the assays of juvenile hormone activity we used freshly molted last instar larvae of *Pyrrhocoris apterus* L. and *Neodysdercus intermedius* Dist. obtained from laboratory rearings. The compounds tested were applied topically to the uninjured cuticle of the abdominal tergites in a constant 1-μl. drop of acetone solution. Five to ten specimens were used in each assay. The hormonal activity was scored according to the degree of preservation of the larval epidermal structures after the final molt. The activity is expressed according to the 0–5 scale introduced by Williams and Sláma (1966). Zero indicates the formation of perfect adults; 3 indicates the formation of individuals with half-larval, half-adult mixture of epidermal patterns; 5 indicates maximum activity as signaled by the formation of perfect extra-larval instars. Each specimen was scored individually and the average was approximated to the nearest unit of the scale. The dose applied to each individual in 1 μl. acetone ranged from 0.001 to 100 μg. The materials were not toxic even when used in high concentrations.

The compounds were synthetized in our laboratories, as described elsewhere

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[1] Institute of Entomology, Czechoslovak Academy of Sciences, Prague. Acknowledgment is gratefully given to Prof. C. M. Williams who provided helpful comments on the typescript and to Mrs. Piščáková and Mrs. Pichlová for their technical assistance.

(Suchý and Šorm, 1967; Czechoslovak patent No. PV 4648-67, PV 4548-67, PV 4709-67, PV 4391-67, PV 6641-67). As a starting material we used p-halogenated benzyl alcohols which were converted to the appropriate lithium derivatives. These were condensed with aliphatic ketones to yield combined aliphatic-aromatic tertiary alcohols (Fig. 1, compound I). The methanol group of the aromatic nucleus was oxidized to give the corresponding acid which was further esterified. The remaining tertiary alcohol group was split off to obtain p-(1,5-dimethyl-1,5 hexadienyl) benzoic acid methyl ester (Fig. 1, II), which we used for the preparation of most of the analogues. The purity of the compounds was checked by elemental analysis and infrared spectroscopy.

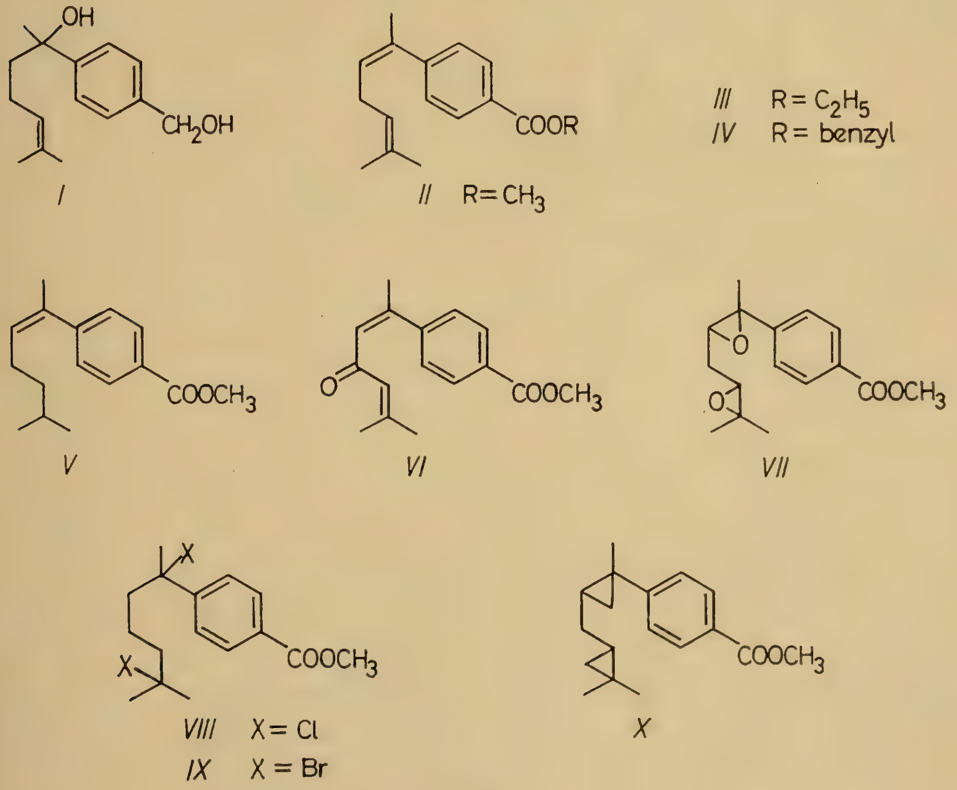

FIGURE 1.

RESULTS

Because the synthetic compounds possess the same carbon skeleton as does dehydrojuvabione and since the latter is known to act mainly on insects of the family *Pyrrhocoridae,* we tested their biological activity on two representatives of this family, *i.e., Pyrrhocoris apterus* and *Neodysdercus intermedius.* The effects were compared with the activity of juvabione and dehydrojuvabione.

1. *Juvenile hormone activity for Pyrrhocoris*

The results, summarized in Table I, show that 7 of the 10 compounds (Fig. 1) were more active than juvabione. The lowest activity was found in compound I, where the primary hydroxyl group was neither oxidized to the acid nor esterified. By contrast the methyl ester (II) exhibited pronounced juvenile hormone activity, which was about 10 times higher than that of juvabione. The ethyl ester (III) was slightly less active than juvabione whereas the benzyl ester (IV) was slightly more active. This indicates that the ester group is essential for juvenile hormone activity and that the methyl ester is the most active.

When analyzing the effect of the substituents in the aliphatic side chain, we therefore used the methyl ester (II) as starting material. Compound V, which is a dihydro derivative of the methyl ester (II), had approximately 10 times lower activity than II. The introduction of a keto group into the aliphatic side chain of II to form compound VI did not lead to further increase in activity. Though this latter substance resembles dehydrojuvabione, it is nearly two orders of magnitude more active than dehydrojuvabione. This finding emphasizes the importance of conjugated system of double bonds in compounds of this type.

Table I

Juvenile hormone activity in topical tests on Pyrrhocoris

	(Dose in μg. applied in 1 μl. acetone)				
	0.01	0.1	1	10	100
Compound I	0	0	0	0	1
II	0	1–2	4	5	5
III	0	0	1	5	5
IV	0	1	4	5	5
V	0	0	1	5	5
VI	0	1	4	5	5
VII	0	0	3	5	5
VIII	0	2	5	5	5
IX	0	2	5	5	5
X	0	2	5	5	5
Juvabione*	0	0	2	5	5
Dehydrojuvabione*	0	0	0	3	5

* Isolated from the wood of balsam fir (Černý *et al.*, 1967). (Each assay was made with 5–10 freshly molted last instar larvae.)

In further studies we investigated the effects of substituents across the two aliphatic double bonds of the methyl ester (II). The introduction of oxirane rings (compound VII) was followed by a slight decrease of juvenile hormone activity. Additions of hydrogen chloride (compound VIII), or hydrogen bromide (compound IX), or methylene bridges (compound X) caused a slight increase in activity.

2. *Juvenile hormone activity for Neodysdercus*

Assays performed on the second species of pyrrhocorid, *Neodysdercus intermedius,* confirmed the substantial juvenile hormone activities of all the synthetic materials except compound I. Indeed, several of the materials were more active for *Neodysdercus* than for *Pyrrhocoris*—for example, compounds V, VI, VII, VIII, IX and X. Compounds VIII and X were nearly 10 times as active for *Neodysdercus* (Table II). And, as in the case of the assays performed on *Pyrrhocoris*, nearly all the materials were substantially more active than juvabione or dehydrojuvabione; Compounds VIII and X were nearly 100 times as active.

3. *Juvenile hormone activity on certain other species*

Topical tests for juvenile hormone activity were also performed on freshly molted last instar larvae of the pentatomid bug, *Graphosoma italicum* Müll. None of the compounds had any significant juvenile hormone activity for this species. Four compounds (IV, VI, VIII and X) showed a trace of activity when the enormous dose of 100 μg. was applied.

TABLE II

Juvenile hormone activity in topical tests on Neodysdercus

	(Dose in μg. applied in 1 μl. acetone)					
	0.001	0.01	0.1	1	10	100
Compound I	0	0	0	0	0	0
II	0	0	2	5	5	5
III	0	0	0	1–2	5	5
IV	0	0	1	4	5	5
V	0	0	0	4	5	5
VI	0	0	4	5	5	5
VII	0	0	1–2	5	5	5
VIII	0	1	5	5	5	5
IX	0	1	5	5	5	5
X	0	1	5	5	5	5
Juvabione*	0	0	0	1	5	5
Dehydrojuvabione*	0	0	0	2	5	5

* Isolated from the wood of balsam fir (Černý *et al.*, 1967). (For further explanation see Table I and in the text.)

Each of the compounds listed was also tested by topical application to last instar larvae of *Acheta domesticus* and *Locusta migratoria* (Orthoptera). Other assays were carried out on freshly molted pupae of *Tenebrio molitor* and *Leptinotarsa decemlineata* (Coleoptera) and *Galleria mellonella* (Lepidoptera). All materials were inactive at dose levels up to 100 μg.

DISCUSSION

The results indicate that the juvenile hormone activity of these aromatic juvabione analogues is dependent on: (1) the chemical nature of the ester radical; (2) the number of the double bonds in the aliphatic side chain; and (3) the substituents in the aliphatic side chain. Similar relationships between the chemical structure and the juvenile hormone activity have also been found among the farnesenic acid derivatives by Sláma *et al.*, (unpublished data). In contrast to farnesenic acid derivatives, where an addition of hydrogen chloride into the molecule increases the juvenile hormone activity for *Pyrrhocoris* as much as 10,000 times (Romaňuk *et al.,* 1967), the chlorinated derivative of p-substituted benzoic acid (compound VIII) is followed by only a minor increase in juvenile hormone activity over the ester II. Bowers *et al.,* (1965) have reported an increase of juvenile hormone activity by the introduction of an oxirane ring into the molecule of methyl farnesoate. In our materials the introduction of these rings, as in VII, failed to enhance the activity of the methyl ester (II). If we compare the biological activity of the methyl ester of farnesenic acid with that of p-substituted benzoic acid methyl ester (compound II), we see that the activity of the latter surpasses that of the former by two or three orders of magnitude (cf. Romaňuk *et al.,* 1967).

The derivatives of farnesenic acid have generally been found more active in juvenile hormone tests performed on *Pyrrhocoris* and less active on *Neodysdercus* (Sláma *et al.,* unpublished data). The results presented here show that the new compounds are usually more active on *Neodysdercus* despite their large size (average weight of freshly molted last instar larvae is 15 mg. for *Pyrrhocoris* and 38 mg. for *Neodysdercus*).

From the biological point of view it is of special interest that the new compounds mimic the juvenile hormone only of insects of the family *Pyrrhocoridae.* This is documented by the fact that the pentatomid species *Graphosoma italicum* is not affected by the topical application or injections of large doses of these substances. Moreover, observed differences in the sensitivities of *Pyrrhocoris* and *Neodysdercus* suggest that the specificity of these effects can exist among members of one and the same family and, as will be communicated later, even among species of the same genus.

Some of the compounds, especially VI, VIII, IX, X, promise to be effective and selective pesticides against the cotton feeding bugs *Dysdercus* and *Neodysdercus,* which have appeared to be sensitive to the "paper factor" (Saxena and Williams, 1966). The new compounds are non-toxic materials with selective action on the *Pyrrhocoridae* and without any significant effects on other insects. The suitability of such materials for insect control has recently been emphasized by Williams (1967).

SUMMARY

1. Certain derivatives of p-(1,5-dimethylhexyl) benzoic acid show high juvenile hormone activity in topical tests performed on larvae of *Pyrrhocoris* and *Neodysdercus* (Hemiptera, Pyrrhocoridae). Some of the newly synthesized compounds are about 100-fold more active than the previously known agents, juvabione and dehydrojuvabione. All these materials are active on insects of the family Pyrrhocoridae and are inactive when tested on other hemipterans or on pupae of Coleoptera and Lepidoptera.

2. It is suggested that these materials may be used to control bugs of the genera *Dysdercus* and *Neodysdercus,* both of which are notorious pests of the cotton plant.

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LACK OF CORRELATION OF SOME PROECDYSIAL GROWTH AND DEVELOPMENTAL PROCESSES IN THE CRAYFISH[1]

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Drach (1939), in a study of the brachyuran crabs *Cancer* and *Maia,* subdivided the premolt period into four substages. In 1944, he further refined his own stages while working with a natantian, *Leander,* by subdividing stage D_1 into three substages. These divisions of the premolt period have been redescribed for use in studies of premolt events in other crustaceans by Hiatt in a study of a grapsoid brachyuran (1948); by Scheer in a study of three natantians (1960); by Kurup in a study of an anomuran (1964); by Travis in studies of two macrurans (1960a and 1965); by Skinner in a study of a brachyuran (1962); by Charniaux-Legrand in a study of some amphipods (1951); and by Stevenson in a study of an isopod (1961 and 1964). Table I compares the designations assigned to some of the premolt events by these workers. This table clearly shows that these events occur at different times in relation to each other in the various crustaceans studied; therefore, it seemed worthwhile to investigate the interrelationships of some of these events in the crayfish *Orconectes sanborni.*

Materials and Methods

Crayfish of the species *Orconectes sanborni* were collected in Plum Creek and in the Cuyahoga River near Kent and maintained in the laboratory in 5-inch-deep running tap water, regulated at 21 ± 3° C. in 22 × 30 inch acrylic plastic aquaria, each divided into six compartments by perforated plastic sheets. This aquarium system was modified after Friberg and Travis (1962). The animals were fed four days a week, canned cat food and Purina rabbit chow two days and uncooked oatmeal and Purina rabbit chow two days. The bottom of each aquarium was covered with oyster shell, made available as a source of calcium, and with white sand.

The intermolt stages of the animals were determined as described by Drach (1939, 1944). Sections of the gastric region of the carapace (Fig. 1), fixed in Zenker's fluid, of five animals in intermolt stage C_4 and at least two animals in each D_1 substage were made and stained with hematoxylin and eosin to determine the degree of reabsorption of old cuticle and development of new cuticle. Animals of all ages collected except those younger than six months were used. The gastro-

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TABLE I

Stage designations assigned to premolt events in different crustaceans

	1	2	3	4	5	6	7	8	9	10	11
Membranous layer present	C_4					C_4		C_4			
Activation of epidermis & hepatopancreas								D_0			
Regenerating limb buds begin rapid growth										D_0	
Gastroliths begin to be formed						D_0				D_0	
Epidermis separates from old cuticle				D_1'a	D_0	D_2	D_0	D_0		D_1	
New setae begin formation as epidermal invagination	D_1	D_1'	D_1	D_1'a	D_1'		D_1'	D_1'			C_4
New setal invagination reaches maximum depth	D_1	D_1''	D_1	D_1''	D_1''		D_1'''				D_2
All morphological details of new setae now visible	D_1	D_1'''	D_1	D_1'''	D_1'''		D_2	D_1'''	D_2		D_4
Reabsorption of membranous layer begins	D_1 (late)					D_2				D_1	
Epicuticle secreted	D_1	D_2		D_2	D_2'	D_2		D_1	D_1''	D_2 (early)	
Secretion of pre-exuvial endocuticle begins	D_1	D_2		D_2	D_2'	D_2		D_2		D_2 (late)	
Most of pre-exuvial endocuticle secreted	D_2	D_2	D_2	D_2	D_2''	D_2		D_2			
Most of the old post-exuvial endocuticle reabsorbed	D_3	D_3	D_3			D_2 $+D_3$		D_3			
Line of dehiscence open	D_4	D_4	D_4	D_4	D_3			D_4			

1. Brachyura: *Cancer, Maia.* Drach, 1939.
2. Natantia: *Leander.* Drach, 1944.
3. Brachyura: *Pachygrapsus.* Hiatt, 1948.
4. Amphipoda: 22 genera. Charniaux-Legrand, 1951.
5. Amphipoda: *Orchestia.* Charniaux-Cotton, 1957.
6. Macrura: *Panulirus, Orconectes.* Travis, 1960a, 1965.
7. Natantia: *Leander, Processa.* Scheer, 1960.
8. Review. Passano, 1960.
9. Isopoda: *Armadillidium.* Stevenson, 1961, 1964.
10. Brachyura: *Gecarcinus.* Skinner, 1962.
11. Anomura: *Petrolisthes.* Kurup, 1964.

lith and carapace lengths of each animal killed to make sections were measured.

Twenty-two animals less than one year old (carapaces 1.5–2.1 cm. long) of both sexes were made to autotomize the right posterior pereiopod during stage C_3 or C_4 (carapace rigid; no sign of premolt). The animals were separated into aquarium compartments, four animals to a compartment. Both the length of the regenerating limb bud and the molt stage of Drach were determined daily for each animal. Measurements were made with calipers reading to 0.05 mm. Fifteen older animals (carapaces 2.0–3.4 cm. long) were made to autotomize the right posterior pereiopod within one day after molting, and the length of the regenerating limb bud and the molt stage of these were determined daily in the same way. Stage D_2 was not looked for in either experiment because the animal must be injured to determine this stage.

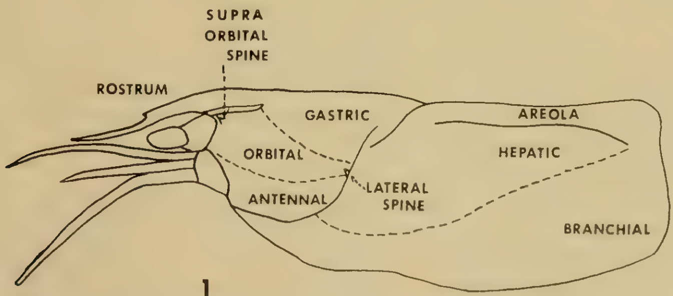

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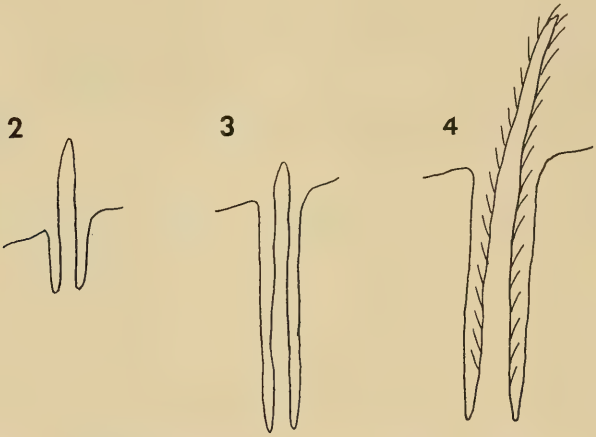
FIGURE 1. Side view of the cephalothorax of the crayfish. The adjectives refer to regions: gastric region, hepatic region, etc. Terminology was taken from Miner (1950, plate 156) and Hobbs (1942).

RESULTS

Criteria for recognizing, in the crayfish, the subdivisions of the intermolt cycle as defined by Drach

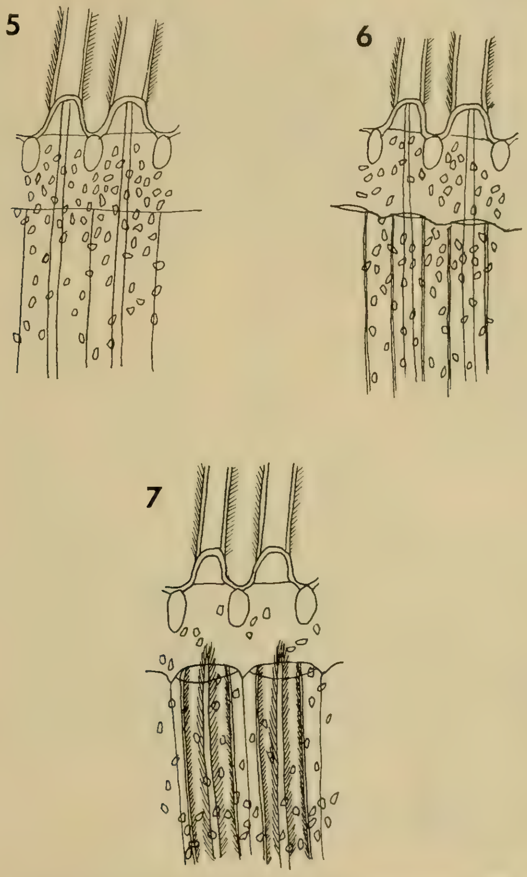
It was found that the crayfish is an easy animal in which to recognize the initial premolt stages as defined by Drach (1939, 1944). These premolt stages begin with stage D_1, in which new setae destined to become part of the new exoskeleton develop. We found that the developing setae of the crayfish can be seen by wrapping the animal in wet cloth or paper tissue with the tail protruding, placing it on a microscope slide on the stage of a microscope, covering the tail with a second slide, and examining the edge at a magnification of 100 ×.

In *Orconectes,* new setae do not appear to develop in quite the way that they do in the natantian *Leander* (Figs. 2–14). In *Leander,* a circular fold or invagination forms at the base of each seta, and the tissue at the base of the seta retracts slightly from the cuticle (stage D_1'). This invagination deepens, eventually reaching a maximum depth, when secretion of the cuticle of each new seta begins (stage D_1''). Soon secretion of the cuticle of the new setae is completed to the extent that morphological details, such as barbules, are visible (stage D_1''') (Drach, 1944). In *Orconectes,* a cylindrical zone with lower cell density than that of the surrounding tissue can barely be discerned extending inward from each seta at the edges of the uropods and telson in intermolt stages A, B, and C (postmolt and intermolt) (Figs. 8 & 9). Instead of forming by invagination of a circular fold, the new seta seems to form by secretion of a tube in the center and a tube at the edge of this zone (Figs. 6 & 11). The two tubes are connected at the bottom, and the inner tube becomes narrower at the top, so that the inner tube becomes the distal half of the new seta and the outer tube becomes the proximal half. Although setae do not seem to form in the same way in *Leander* and *Orconectes,* it was possible to subdivide stage D_1 in *Orconectes* on the basis of essentially the same



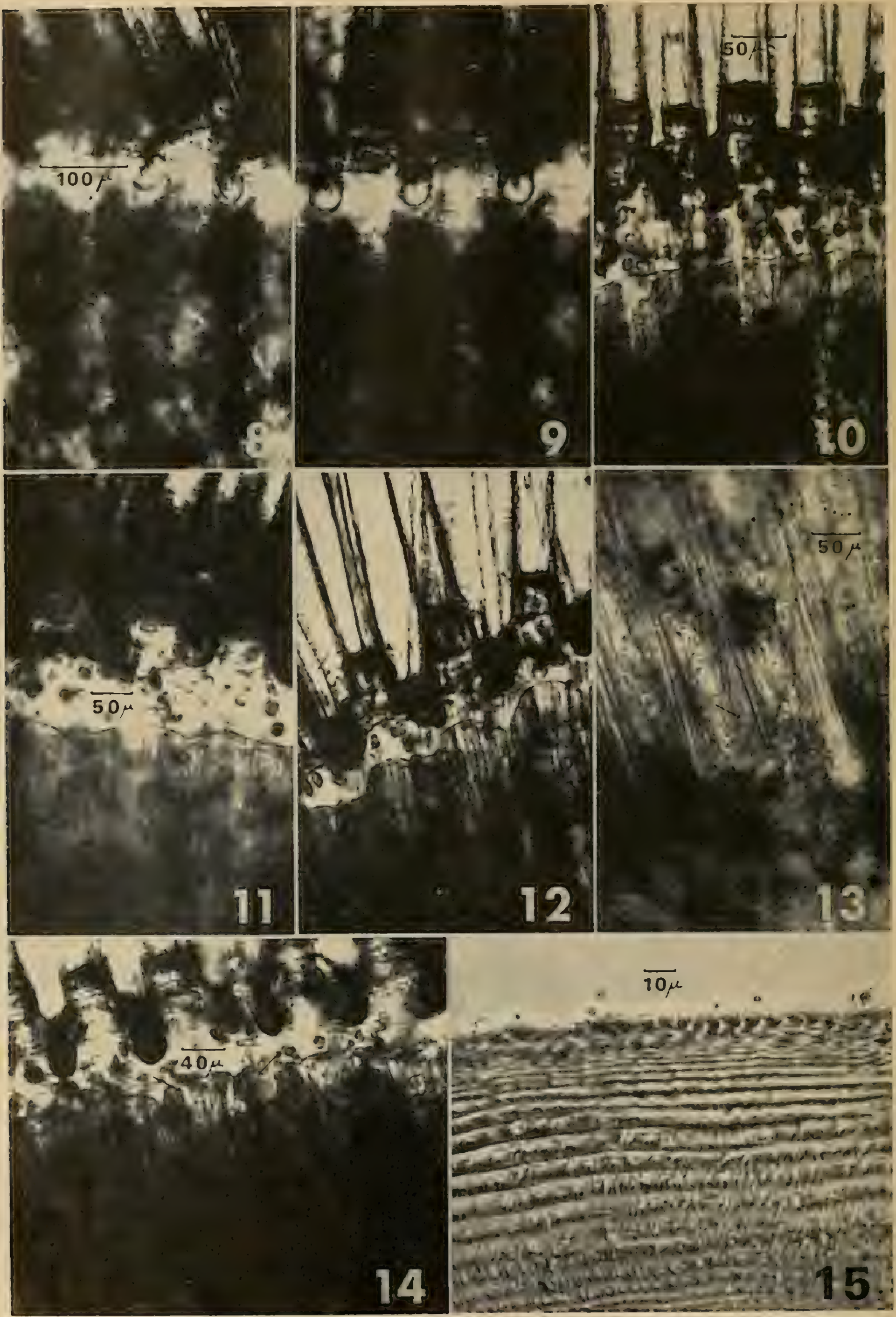
FIGURES 2, 3 AND 4. Development of setae in the Natania as described by Drach (1944). Figure 2. Stage D_1'. Figure 3. Stage D_1''. Figure 4. Stage D_1'''.

criteria as used by Drach to subdivide stage D_1 in *Leander*. In *Orconectes*, formation of new setae at the edges of the uropods and telson begins when the epidermis retracts, forming a sharp line a short distance below the cuticle (Figs. 5 & 10). The retraction of tissue places this event in stage D_1'. Next, the tube within a tube described above appears and at the same time the sharp line below the cuticle mentioned above becomes indented around each new seta (Figs. 6 & 11). The appearance of the tubes is caused by the beginning of secretion of the cuticle of the new setae, which places this event in stage D_1''. Stage D_1''' begins when fine, hairlike branches appear on each seta (Figs. 7, 12, 13, & 14).



FIGURES 5, 6 AND 7. Development of setae at the borders of the uropods and telson of the crayfish. Figure 5. Stage D_1'. Figure 6. Stage D_1''. Figure 7. Stage D_1'''.

Later stages in *Orconectes* can be recognized by exactly the criteria described by Drach (1939). Stage D_2 can be recognized by removing a piece of old cuticle or by pulling off a dactyl to see the new cuticle underneath, but not by examining the new setae, which look no different from their appearance when the barbules first appear in stage D_1'''.


100μ
8
9
50μ
10
50μ
11
12
50μ
13
40μ
14
10μ
15

Figures 8–15.

In stage D_3, resorption of the old cuticle is in an advanced state, and the line of dehiscence will break under finger nail pressure (Drach, 1939). Because much of the old endocuticle has been reabsorbed, the entire body of the crayfish can readily be compressed somewhat between the fingers.

Stage D_4 is characterized by the opening of the line of dehiscence. Reabsorption of the inner layers of the old cuticle is now complete (Drach, 1939). The crayfish cuticle splits transversely immediately behind the carapace a short time before ecdysis.

Cuticle sections

In stage C_4, the cuticle is complete, including the inner, noncalcified membranous layer, which consists of laminae as thin as or thinner than 1 μ. A histochemical test for calcium is required to delimit the boundary between the membranous layer and the rest of the endocuticle because there is no sharp distinction between thin and thick laminae; instead, the thickness of the laminae progressively increases toward the outside. At this stage the inner edge of the cuticle was found to be smooth and the epidermis was thin and tightly attached to the cuticle.

At the beginning of stage D_1 (stage D_1') the epidermis had grown considerably thicker and had separated from the cuticle. Furthermore, there was evidence that reabsorption of the cuticle had begun in all sections examined in stage D_1 (Fig. 15). The inner edge of the cuticle was rough or wavy or several of the inner laminae were splitting away from the rest of the cuticle. One might expect to find progressively more reabsorption of the membranous layer in stages D_1'' and D_1''' than in stage D_1', but such was not the case. Instead, laminae as thin as or thinner than 1 μ were found to be completely absent (reabsorbed) in about the same proportion of specimens, half, in each D_1 substage.

No laminae as thin as 1 μ were seen in any of the specimens examined in stages D_2 and D_3, and by stage D_3 much additional endocuticle had been reabsorbed. In both specimens fixed at the beginning of stage D_3, about half of the new preexuvial cuticle had been secreted; therefore, about half the preexuvial cuticle must be secreted in stage D_2 and the other half in stage D_3 and D_4.

Gastroliths

Gastroliths are absent in stage C_4 and present in all substages of stage D. Within stage D_1, one might expect the ratio of gastrolith to carapace length to increase progressively from stage D_1' to D_1'' to D_1'''. Instead, the following ratios ($\times$ 100) were found: in stage D_1', 10, 11, 11; in stage D_1'', 9, 9, 10, 13; in stage

FIGURES 8 AND 9. Two views of edges of crayfish uropods at stage C, showing regions of low cell density extending inward from each seta.

FIGURE 10. Edge of crayfish uropod at stage D_1', showing the edge of the epidermis, which has pulled away from the old cuticle.

FIGURE 11. Edge of crayfish uropod at stage D_1''. Formation of new setae has begun with the secretion of a tube within a tube underneath each old seta.

FIGURES 12, 13 AND 14. Three views of fully developed setae in crayfish uropods at stage D_1'''. Arrows indicate some of the barbules. Figure 13. Proximal ends of new setae.

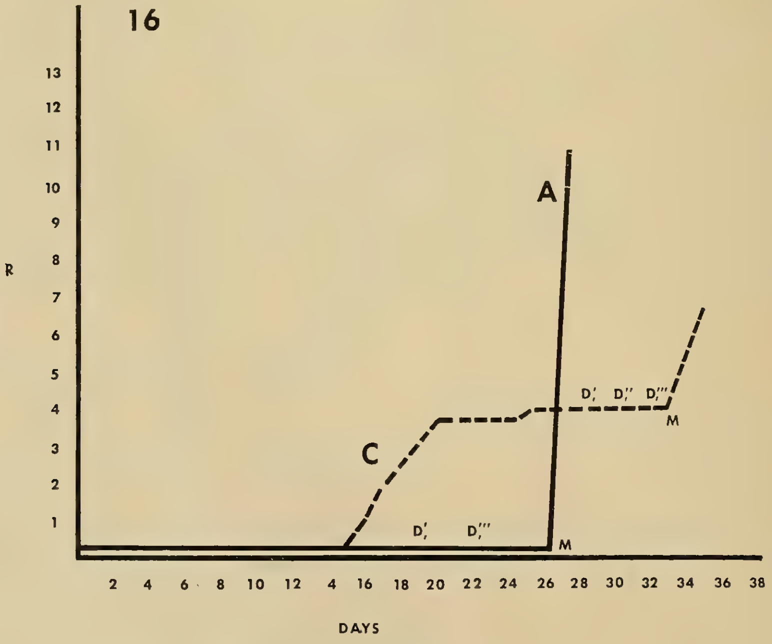
FIGURE 15. Rough inner edge of the old cuticle at stage D_1'. The inner edge is at the top of the photograph. Iron hematoxylin and eosin. Phase contrast.

Table II

Types of growth curves of regenerating limb buds

Type	Frequency		Description
	1st experiment	2nd experiment	
A.	10	2	Little or no rise until ecdysis, when there is sharp rise.
B.	1	1	Slight rise, plateau, rise, plateau, rise until ecdysis.
C.	4	8	Slight rise, plateau, rise, plateau until ecdysis.
D.	2	3	Slight rise, plateau, rise until ecdysis
E.	2	0	Rise, plateau, rise until ecdysis.
F.	3	0	Rise, plateau until ecdysis.
G.	4	0	Rise without plateau.

D_1''', 11, 11, 13. The means of these values are 10.7, 10.3, and 11.7, respectively. If one ignores the D_1 substages and looks to see whether the gastrolith : carapace ratios vary according to whether absorption of the membranous layer is complete or not, one finds the following ratios: membranous layer present, 9, 9, 10, 13; membranous layer absent, 10, 11, 11, 11, 11. The mean values are 10.3 and 10.8, respectively. Therefore, gastrolith : carapace ratios do not seem to vary significantly with absorption of the membranous layer.



Figures 16, 17 and 18. Representative growth curves of regenerating limb buds. The beginning of each premolt stage or substage is indicated on the curves. For a definition of "R," see text.

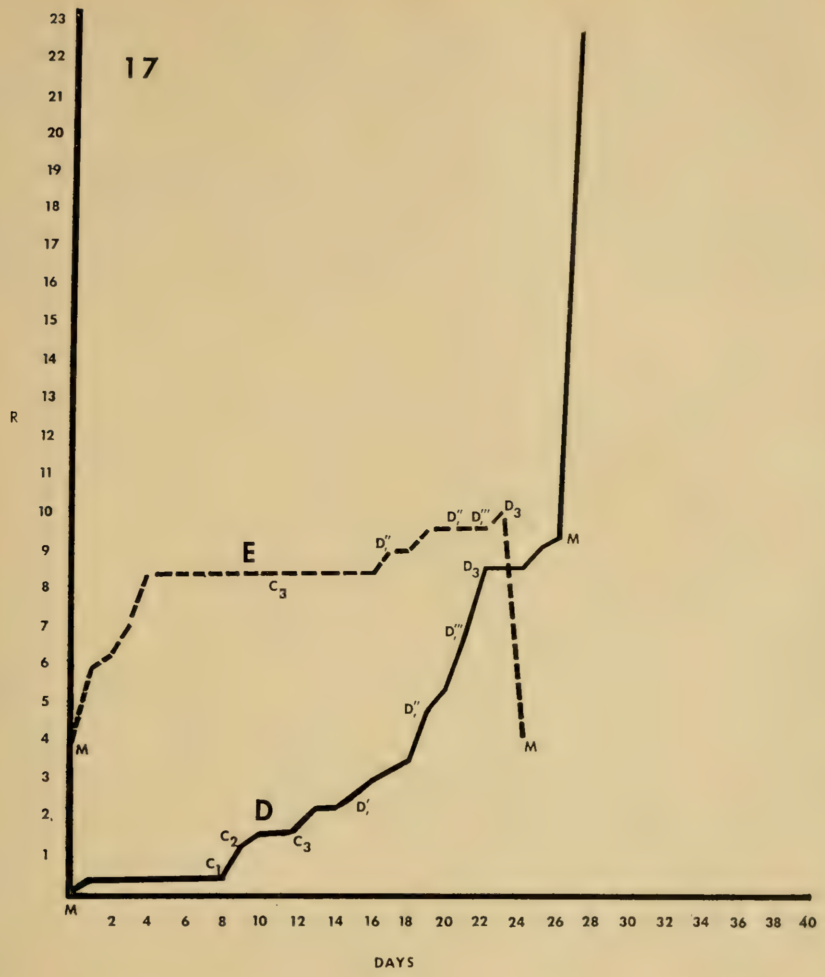


Figure 17.

The gastrolith : carapace ratios found in later stages were: in stage D_2, 13 and 16; in stage D_3, 17 and 19; and in stage A_1 (immediately after the molt), 18 and 19. Thus, the ratios increased progressively after stage D_1, and by stage D_3, they reached values equal to those found immediately after ecdysis.

Regenerating limb buds

The right posterior pereiopod was removed during stage C_3 or C_4 in the first experiment. Three of the 22 animals died before reaching stage D_1', but all the others molted at least once, and there were 26 molts.

Seven types of growth curves were obtained when R was graphed against time in days (Table II). (R = 100 times the length of the regenerating limb bud divided by the carapace length. [Bliss, 1956; Durand, 1960]). These different types of curves will be designated by letters. In type A, exhibited in 10 cases, there was little or no rise in the R value until ecdysis, when R rose sharply (Fig. 16A). This type of curve was produced by regeneration of the limb bud inside the base of the old limb instead of protruding from the base. Such regeneration was invisible until liberated at ecdysis.

Three other types of curves began with a very slight rise in R followed by a plateau lasting 5 to 15 days. Then there was a further, much greater rise in R. The three types differed in their shapes after the beginning of this rise. In type B, exhibited in one case, there was a second plateau lasting 11 days before a final

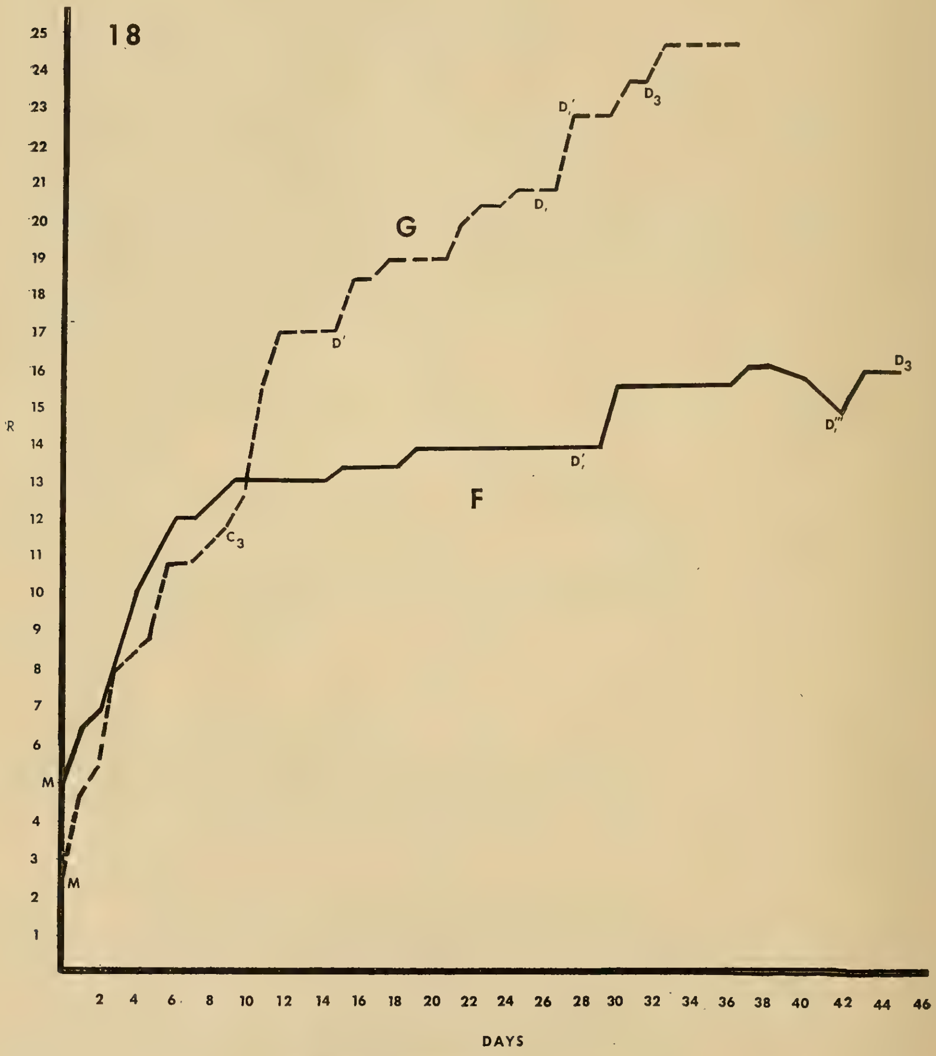


Figure 18.

rise in R before ecdysis. Formation of setae in the uropods and telson began during this second plateau. In type C, exhibited in four cases, there was also a second plateau, which, however, persisted until ecdysis (Fig. 16C). Thus, in this type also, seta formation began during a growth plateau. In the third type, type D, once the curve began to rise, the rise continued more or less steadily until ecdysis without a second plateau (Fig. 17D), and seta formation began during this rise. Two growth curves were of this latter type.

Three types of curves began with greater immediate rises than those of types B, C, and D. Of these, type E, found in two cases, also displayed a plateau followed by a steep rise preceding ecdysis (Fig. 17E), during which new setae began to form. Type F, exhibited in three cases, was the same as E, but lacked the proecdysial rise (Fig. 18F). In these, therefore, new setae began to form during a growth plateau. Finally, there were four animals that displayed type G, in which growth was almost continuous from the day regeneration began until ecdysis (Fig. 18G).

TABLE III

R values at day before molt arranged in order of number of days of first molt after leg removal

First Experiment				Second Experiment	
First molt		Second molt		First molt	
Days after leg removal	R	Days after leg removal	R	Days after leg removal	R
9	4.60	37	3.24	19	18.97
10	.31	33	10.23	22	15.89
11	.26	47	25.60	25	4.76
11	.28	33	16.67	27	23.67
12	.30	34	11.30	28	1.37
14	.28	44	11.71	28	1.59
18	3.68	45	13.00	29	15.97
20	5.43	47	12.44	30	6.03
21	3.03	66	16.11	31	24.83
23	.30			33	2.43
24	.00			38	2.52
32	.27			38	15.57
33	.24			40	2.55
33	.29				
33	3.89				
35	10.95				
38	.26				
47	.97				

Figure 18F illustrates a phenomenon that occurred several times during the study. At one point, the R value actually decreased. A few times when this occurred there was a dark scab at the tip of the regenerating limb bud. It may be that one of the other crayfish took a bite and so shortened the limb. Probably all the decreases in R were due to injury.

To summarize, there was almost no visible regeneration during 38.5% of the 26 intermolt periods studied, and in the others, regeneration began with a very slight rise in R in 27% of the 26 intermolt periods, and it began with a greater rise in R in 34.5%.

It was hoped that R would always reach about the same value at ecdysis so that the R value could be used to predict the time of ecdysis in the crayfish as it has been used in other crustaceans. However, the R values in this experiment varied widely, as shown on Table III. Since R increases greatly immediately after ecdysis, the values obtained the day before were compared. As shown on the table, R varied from 0 to 10.95 one day before the first molt after removal of the pereiopod, and there was no correlation between the length of time available for regeneration and the magnitude of R except that almost all animals molting within 14 days after leg removal had small R values at ecdysis. As would be expected, R values were larger at the second molt after leg removal (Table III).

The values of R also could not be used to predict the time when formation of new setae begins. The value of R at this time minus the value of R at the previous molt, if any, varied from 0.5 to 15, not including R values of type A curves, and one individual reached a value of 16.5 without beginning new seta formation when the experiment was discontinued.

If a sharp rise in R value greater than 1 is considered to be "proecdysial limb growth" (Bliss and Boyer, 1964), then the formation of new setae always began after the beginning of proecdysial limb growth unless there was no such growth. The length of time after varied from 0 to 28 days.

Larger crayfish, which have a longer intermolt period, were used in the second experiment. More time for basal limb growth was also obtained in the second experiment by removing the right posterior pereiopod earlier in the cycle, that is, within one day after a molt. Then each animal was studied until the next molt. Fourteen growth curves were obtained from the 15 animals; one animal died before molting.

In this second experiment, two animals exhibited regeneration pattern A, one exhibited pattern B, 8 exhibited pattern C, and three exhibited pattern D. None of the growth curves in this experiment followed patterns E, F, or G.

Thus, there was almost no visible regeneration during 14.3% of the 14 intermolt periods. All the remainder of the growth curves began with a slight rise in R followed by a plateau.

As shown on Table III, R varied from 1.37 to 24.83 at ecdysis. The R values at the first molt after leg removal were higher, on the average, in the second experiment than in the first. R also varied widely, from 5 to 15.5, on the day when seta formation began.

As in the first experiment, the formation of new setae always began after the beginning of proecdysial growth as defined above unless there was no proecdysial growth. The length of time after varied from 5 to 22 days.

Durations of the premolt stages

The data from the experiments on regenerating limb buds were combined to obtain the following means ± standard errors: stage D_1' ($n = 22$), 5.3 ± 0.6 days;

stage D_1'' ($n = 27$), 3.3 ± 0.3 days; stage D_1''' and stage D_2 together ($n = 26$), 2.4 ± 0.2 days; stage D_3 ($n = 25$), 3.0 ± 0.3 days. Stage D_4 lasts an hour or two.

Discussion

On the basis of the work reported here, it is now possible to say in which substages of stage D certain premolt events occur in *Orconectes sanborni.* These are events which, as shown on Table I, seem to occur in different substages in different crustaceans. In this study, the beginning of formation of new setae was taken as the beginning of stage D_1, following Drach (1939, 1944). It was found that the membranous layer of the cuticle begins to be reabsorbed, the epidermis separates from the old cuticle, and gastroliths begin to be formed, all before the beginning of stage D_1; that is, these three events had already occurred in all specimens examined in stage D_1. Therefore, in *O. sanborni,* these events must be assigned to stage D_0, which cannot be distinguished by any external changes in the animal (Travis, 1960b; Passano, 1960; Skinner, 1962). New setae of regions of the body other than the uropods and telson were not examined. It is possible that the setae of other regions form at different times in relation to the other premolt events studied. It is also possible that in regions other than the gastric region the epidermis may separate from the cuticle and the membranous layer may be reabsorbed at different times in relation to the formation of the setae. It is known that the setae of different parts of the body form at slightly different times and that cuticle secretion is also not fully synchronized in different areas (Drach, 1939).

Drach was ambiguous in his definition of stage D_2, and this has lead to some confusion (see Table I). It is not clear whether one should place the beginning of secretion of new cuticle in stage D_1 or stage D_2. Drach said (1939, p. 190) that stage D_2 begins only when enough new cuticle is present to be visible to the naked eye; therefore, the epicuticle and some procuticle are secreted in stage D_1. However, he also said (1939, p. 187) there is no difference in the general integument between stages C_4 and D_1 except for the beginning of gelification of the inner layer of the cuticle in stage D_1. This implies that secretion of the new cuticle does not begin until stage D_2. In his 1944 paper he also says that none of the new general cuticle is secreted yet in stage D_1, and most workers have followed this statement in their use of Drach's stages. We feel that precedent now demands that stage D_2 be defined as beginning when secretion of new epicuticle begins, even though it may be impossible to determine precisely when this occurs in the living animal. Of course, this definition would not apply to any crustaceans in which the epicuticle is secreted before or during the formation of new setae (see Stevenson, 1961 and 1964).

Frequent observations of seta formation in the same animal were made possible by our discovery that developing setae of the uropods and telson could be seen without injury to the animal. Developing setae can also be studied *in vivo* in the Natantia (Drach, 1944) and in the Anomura (Kurup, 1964) because transparent parts of these crustaceans are so located that they can be placed under the microscope without injury to the animal. To see the developing setae in the Brachyura, on the other hand, one must cut off part of an epipodite after making a hole in the brachiostegite or, if the animal is a female, part of a pleopod (Drach, 1939).

Gastrolith:carapace ratios were calculated in this study because McWhinnie (1962) had found that this ratio varies in a regular way in *Orconectes virilis.* It progressively increases during premolt and reaches 0.18 to 0.22 at the time of ecdysis. Our findings agree with this. Johnson (1962), on the other hand, found that the sizes of gastroliths of individuals of the same size of *Cambarus longulus longulus* varied at molt. The lack of correlation in our study between rate of gastrolith growth, rate of seta formation, and rate of reabsorption of the membranous layer has its parallel, in a sense, with the finding of McWhinnie (1962) that gastroliths may begin to form in stage C_2 in eyestalkless crayfish. This is an extreme lack of correlation, being a case of gastrolith formation before the old cuticle is even completed.

Regeneration of the right posterior pereiopod in different crayfish was observed to follow seven different patterns, here designated types A–F. In the type of limb regeneration exhibited by the crayfish, no limb bud is externally visible if the limb was severed either distal or proximal to the preformed breakage plane (Bliss, 1960). All animals that exhibited pattern A (Fig. 16A), in which there was no externally visible limb bud, were carefully examined to make certain the limb had been severed at the preformed breakage plane, and all were found to have been so severed. However, during autotomy (actually autospasy, *i.e.,* aided by a pull by the investigator, in some cases), the stump of the limb may have become injured so that the result was the same as a cut proximal to the preformed breakage plane.

Regeneration type D (Fig. 17D) was the most similar to the kind of regeneration described by Bliss and Boyer (1964) and by others. (For references, see Bliss and Boyer, 1964.) There is an initial period of regeneration called "basal limb growth," then a period during which there is little or no growth of the limb bud, called "growth plateau," and finally a period of rapid growth before the molt, called "proecdysial limb growth." In regeneration type D, basal limb growth produces only a slight bulge beyond the breakage plane, or, after the molt, only a slight elongation of the bud. Then there is a growth plateau for some days, followed by proecdysial limb growth continuing until ecdysis. Regeneration types B and C (Fig. 16C) are similar to type D except that they include a second, higher, growth plateau occurring at least partly during proecdysis. This second plateau may be produced by environmental factors or by injury to the bud. Bliss and Boyer (1964) describe several experiments in which environmental factors produced growth plateaus during proecdysis. However, there are two reasons for thinking the second plateaus in our experiments were not produced by factors in the environment that delay proecdysis. First, stage D_1' was observed to begin during the plateau. This shows that proecdysis was progressing and was not being delayed. Second, the plateau persists until ecdysis in type C. This again shows that proecdysis was not being delayed during the plateau.

Regeneration pattern B is similar to the pattern obtained by Durand (1960) during a study of limb regeneration in the crayfish *Orconectes limosa.* Durand found a lag period, comparable to what we have called growth plateau, lasting about six days. Then his crayfish exhibited "basal growth," comparable to what we have called "proecdysial growth," until the value of R was about 22. Then there was a plateau lasting until proecdysial growth, which began three to five days before ecdysis. However, our type B cannot be fitted entirely to Durand's pattern because stage D_1' began during the second plateau, showing that proecdysis had already begun. Skinner (1962) found that proecdysial limb growth takes place during stages D_0 and D_1 in the land crab *Gecarcinus lateralis*. If the same is true in the crayfish, the second plateau exhibited by some of our crayfish takes place in the middle of proecdysis. Furthermore, if the mean lengths of the premolt stages found in this study for *O. sanborni* are added, the total is 14 days. Therefore, three to five days are not enough for proecdysis.

There seem to be three possible explanations for the discrepancy between our results and those of Durand. First, regeneration is different in the two species of *Orconectes*. Second, in both species, growth plateau occurs at a rather high value of R, and proecdysial growth begins at a later stage, probably D_2 or D_3, than in Brachyura. Third, in both species, growth plateau occurs at a very low value of R, and any second plateau is superimposed on proecdysial growth by injury or by some environmental factor. An argument in favor of the third explanation is that curves B, C, and D occurred following the molt after autotomy as well as between autotomy and the succeeding molt. In the former case, one would not expect any basal growth, since this had already occurred during the previous intermolt period, or if another period of basal growth did occur, one would expect it to begin immediately after the molt because there seems no reason to expect a lag during postmolt. More work is needed before one can decide definitely between these three explanations.

Regeneration type G (Fig. 18G) seems to represent a proecdysial rise which occupies the entire growth curve. Types E and F (Figs. 17E & 18F) seem to be derived from type G in the same way as types B and C seem derived from type D. A plateau is added during proecdysial growth. These three patterns would be expected whenever autotomy occurred during or immediately before proecdysis. In fact, type G has been described previously in just this situation (Durand, 1960; Bliss and Boyer, 1964). In our experiments, it was intended that autotomy occur in stage C_3 or C_4, but it may, in some cases, have occurred in stage D_0 since D_0 cannot be distinguished from C_4 by any external morphological criteria. However, in our experiments, regeneration types E, F, and G were never found before the first molt after autotomy. Therefore, they are not due to the occurrence of autotomy during or immediately before proecdysis. Instead, they are examples of rapid growth, which may be interrupted by a plateau, from one molt to the next. This phenomenon must have some significance in terms of the growth potential of the animal, and perhaps it predicts a short duration of stage C_4 (intermolt).

In all cases, stage D_1' began after the beginning of the rise in R value which we are tentatively calling the proecdysial growth. This agrees with the time of beginning of stage D_1 in *Gecarcinus* according to Skinner (1962), as mentioned above. However, the actual value of R at the beginning of stage D_1' in *Orconectes* varies, as does the value of R at ecdysis.

The principal conclusion that can be reached from this study is that certain processes occur during stage D_1 (early premolt) at rates which are independent of each other. These processes are formation of new setae, reabsorption of the membranous layer of the old cuticle and concomitant separation of the epidermis from the cuticle, secretion of gastroliths, and premolt growth of regenerating limb buds. These processes seem to be started by the same trigger, but the rates are under independent control such that one is relatively faster in one individual and another is relatively faster in another individual.

The authors wish to thank Dr. Dorothy Bliss of the American Museum of Natural History for reading the manuscript.

SUMMARY

1. New setae can be seen in the process of development in the uropods and telson of living specimens of *Orconectes sanborni*. The setae form by secretion of a tube within a tube. Three stages in the formation of the new setae can be used to subdivide intermolt stage D_1 into three substages.

2. The beginning of seta formation defines the beginning of stage D_1. Before beginning this stage, the membranous layer of the cuticle begins to be reabsorbed, the epidermis separates from the old cuticle, gastroliths begin to be formed, and the rate of regenerating limb growth increases sharply except in individuals which do not display such increase at all.

3. The membranous layer had been completely reabsorbed in about half the specimens examined in each D_1 substage. Therefore, the rate of reabsorption of the membranous layer is not correlated with the rate of seta formation.

4. Ratios of gastrolith length to carapace length and of regenerating limb bud length to carapace length varied in different specimens, but the variations were not correlated either with D_1 substage and therefore with rate of seta formation or with reabsorption of the membranous layer.

5. Growth curves of regenerating limb buds followed seven different patterns.

6. Mean lengths of premolt stages of animals of 1.5 to 3.4 cm. carapace length were: stage D_1', 5.3 days; stage D_1'', 3.3 days; stage D_1''' and stage D_2 together, 2.4 days; stage D_3, 3.0 days.

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PHOTOPERIOD CONTROL OF WINTER DIAPAUSE IN THE FRESH–WATER CRUSTACEAN, DAPHNIA

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Polymorphism and diapause in arthropods may be evoked by photoperiod, although control is sometimes conditional to a certain range of temperature or an adequate diet (Lees, 1953; Paris and Jenner, 1959; Adkisson *et al.,* 1963; Danilevskii, 1965). Further intricacy of control of terrestrial diapause is provided by the critical photoperiod of the adjustment to provide latitudinal adaptation, and by the number of instars separating the photosensitive and responding stages (Danilevskii, 1965; de Wilde, 1962; Beck, 1963). A recent entry to the list of examples of photoperiodism is the genus *Daphnia,* a fresh-water member of the Cladocera (Crustacea). Expression is reported to be conditional, and with a permissively short photoperiod the culture may shift from parthenogenic to sexual reproduction but only with the provision of a second stimulus which is associated with density of the culture (Stross and Hill, 1965). Details of inception of sexual reproduction and termination of diapause in the overwintering embryos are presented below.

Embryonic diapause in *Daphnia* follows the shift from parthenogenic to sexual reproduction. The sexually derived embryo is structurally conspicuous as are its enveloping (ephippial) membranes. In reality the diapause may be obligatory to the sexually determined embryo, in which case the expression of a polymorphism rather than of diapause is under study. Sexual reproduction in *Daphnia* (and embryonic diapause) has been observed in all seasons of the year (Berg, 1931). Berg, among others, distinguishes three types of life cycles which include shifts to sexuality in both short- and long-day photoperiods. The acyclic type reproduces exclusively by parthenogenesis, and a motile, reproductive diapause has been described for over-wintering individuals (Brooks, 1965). The acyclic type which has been described for the larger, deeper lakes, may retain the capacity for sexual reproduction, however, as Berg (1931) demonstrated. The monocycle contains a single period of sexual reproduction, and this seems to occur primarily if not exclusively in autumn; it is found in small but permanent bodies of water. A third type is the polycyclic, and in Berg's reported observations, it consists mainly of two periods that are spring- and autumn-initiated. The autumn period is reported to be the more intense, and one species (*D. pulex*) may produce only the sexual (ephippial) eggs in some lakes. The polycycle is apparently restricted to ponds where there are at least two periods of "unfavorable" conditions. A single species, *D. pulex,* is reported to exhibit all types of life cycles and to pursue simultaneously both kinds of reproduction with varying degrees of intensity (Berg, 1931).

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METHODS AND MATERIALS

The experiments were performed on two populations of *Daphnia pulex* Leydig about which the natural environment and seasonal life history were known in some detail (see below). Methods for inducing a shift to sexual reproduction (or diapause) have been stated briefly (Stross and Hill, 1965). In essence, they consist of subjecting groups of even-aged individuals to various photoperiods under a standardized set of conditions. Cultures of the population from Paul Lake were started with specifically identified young that had hatched from ephippia in the laboratory. Cultures of *D. pulex* from Peter Lake were begun with adults transported from the lake. They were maintained in (Paul) lake water or a synthetic substitute; each liter of distilled and deionized (Deeminac) water contained in milligrams, $NaHCO_3$ 10.0; KNO_3, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 2.0; $CaCl_2$, 10.0; and $FeCl_3$, 2.9 mg. The cultures were fed suspensions of the flagellate, *Chlamydomonas reinhardti* Indiana U. strain #89 or 90, which had been grown axenically on slants of agar containing nutrients as already described (Stross, 1963).

The influence of photoperiod on the type of reproduction was measured with groups of five females, started when not more than 2 days old, in 20 ml. of medium contained in shell vials (25 × 95 mm.). Each experiment was started from nonclonal stock or experimental cultures. Density was maintained, usually in five replicates, through replacement of losses from companion vials. The influence of density was measured in the same way except that the various densities of *Daphnia* were maintained in 100-ml. beakers containing 50 ml. of medium.

Each replicate was examined at two-day intervals for young and ephippia. At that time the adults were transferred to fresh suspensions of food equal to 1.5×10^5 cells per milliliter. Temperatures were maintained at either 12 or 19° ± 1.0° C. Photoperiods were supplied by time-controlled intervals of "daylight" fluorescent light at an intensity of 1350 lux.

The intensity of sexual reproduction (embryonic diapause) is measured as the ratio of sexual (ephippial) broods to the total (sexual and parthenogenic) produced during the 30 days of an experiment. The number of parthenogenic broods produced in each 2-day interval was estimated from an arbitrary assignment of a number of young per brood for each level of feeding. Occasional inspection of the contents of the brood pouch of the mother indicated the technique to be satisfactory.

The duration of the diapause interval and the environmental control of diapause termination were tested in both laboratory and lake. The ephippia (*i.e.*, embryos and enveloping membranes) were collected on or about 1 November from the shoreline of Paul Lake (see below) and returned to the laboratory in myriad quantity. They were stored (incubated) in lake water at various densities and photoperiods in capped jars. In the densest containers, the mass of floating ephippia measured $\frac{3}{8}$ to $\frac{1}{2}$ inch in thickness, while in the sparsest they were restricted to approximately 2 to 3 layers deep. Aeration of the ephippia was supplied by inverting the container once each day and replacing the medium once each week. The same water was returned to the containers after a week of storage. Storage, or what may be considered incubation, was either at 20.0° C. or at 4.0° C. in constant dark or at specified photoperiods.

The time of diapause termination was measured with duplicate groups of 50 ephippia each, unless otherwise stated, that had been transferred from the larger containers to 50 ml. of (Paul) lake water. The young that hatched from the ephippia exposed to light were removed at intervals of usually one day. The controls were not examined until several days after hatching had begun in light-exposed containers and then aperiodically with the aid of a far-red-emitting safelamp as described (Stross, 1966).

To provide an aerated micro-environment around the floating ephippia, they were placed in glass cylinders under controlled photoperiods and supplied with a gentle stream of water-washed air.

The role of light was also tested in the field. Ephippia were placed in light-baffled cages at several depths in the autumn (approximately 1 November) and lifted at the anticipated time of hatching the following spring. In the first experiment the light control was housed in an opaque cylinder of plastic, which was made light-proof by the attachment of elbows and vertical extensions (Fig. 1a). The transparent cylinder was left open at either end. A refinement in the second experiment permitted equal rates of water flow in both containers. This was achieved by placing light baffles at each end of the tube and these consisted of three overlapping semi-circles of aluminum plate, painted with flat-black acrylate to reduce scattering of light (Fig. 1b). The ephippia were sealed in a Pyrex glass cylinder walled at each end with nylon bolting cloth (#10 mesh) which was held in place by polyethylene expanders. A water-tight vial containing fast and slow panchromatic photographic film was placed in the glass cylinder with the ephippia as a check against light-leaks. The tube containing ephippia was centered inside the cylinders and held in place with plastic toothpicks. After the light baffles were inserted into slits at the ends of the cylinder, the slits and toothpick holes were covered with windings of electrical Scotch Tape.

The day on which diapause terminated was determined by subtracting the interval of post-diapause from the day hatching occurred. A mean length of post-diapause development at each temperature was determined from samples of several hundred embryos at each of four temperatures; it was 3.5 days at 21.0° C., 4.0 days at 17.0° C., 6.7 days at 12.0° C., and 18.0 days at 3.5° C. These determinations were made with embryos that were forced to remain in diapause approximately two months beyond the normal time of termination. The interval of post-diapause development was measured from the time the embryos were transferred to fresh medium.

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Phenology of parent populations

The experiments were performed on stock whose ancestry is known to shift from parthenogenesis to sexual reproduction (*i.e.*, enter diapause) in autumn only. The population in Paul Lake, Michigan (T45N R8ES36; Latitude 45° N), is in transition during the first week of October. In mid-September reproduction is all parthe-

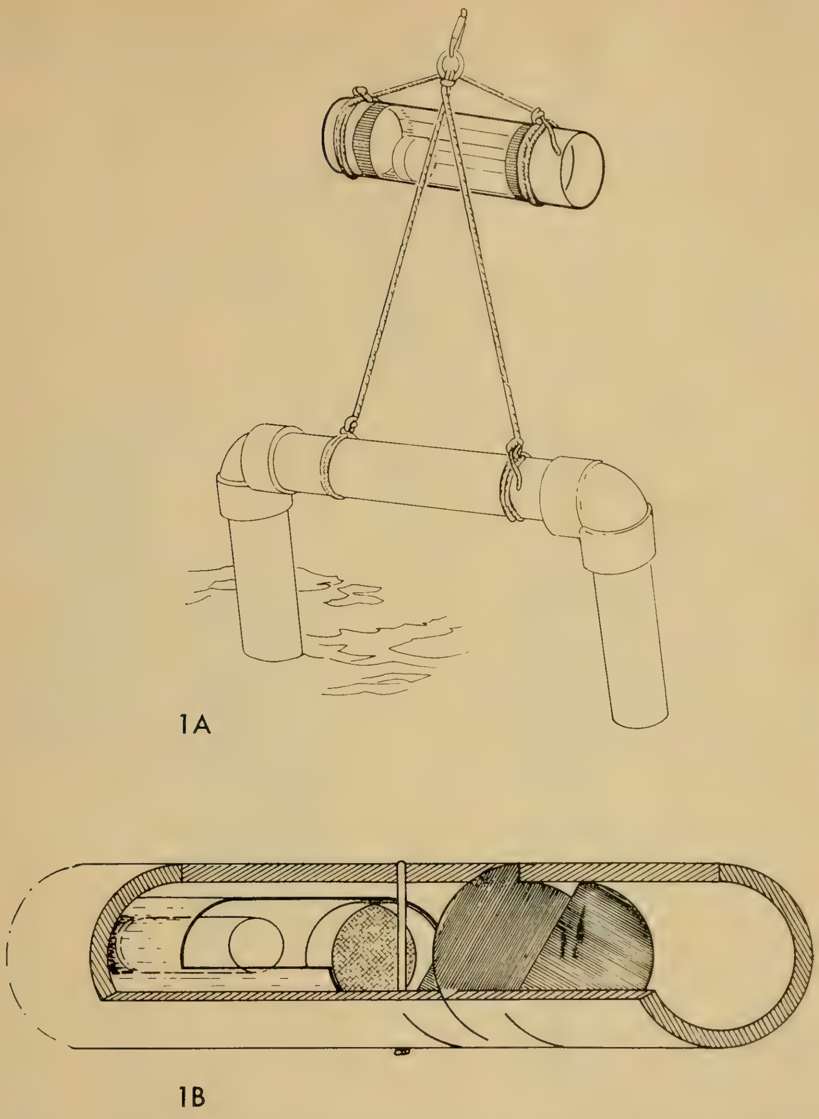

FIGURE 1. Light and dark chambers for *in situ* incubation of diapausing embryos of *Daphnia.* In the first experiment the dark chambers consisted of a tube of opaque plastic with elbows and vertical pieces facing downward (1A). The embryos (ephippia) were contained in a glass tube which was centered inside the outer tube between the two bands of tape. Light baffles were substituted for the vertical tubes in the second experiment (1B). An assembled tube consists of (from left to right) windings of electrical Scotch Tape which cover the baffle ports, photographic film container, chamber containing embryos, nylon bolting cloth held in position by a polyethylene expander, plastic rod which holds the chamber in place, and three light baffles made of aluminum and sprayed with a flat-black acrylate. The transparent chamber consisted of an outer lucite tube in the first experiment (1A) and an inner tube which contained the embryos. In the second experiment (not shown) the outer chamber was fitted with light baffles in order that water exchange in both light and dark chambers would be comparable.

nogenic and by the third week in October it is all sexual (ephippial) (unpublished observations). The magnitude of the effort is apparent from the large masses of ephippia to be found accumulated along one part of the margin of the lake.

Equally predictable is the absence of diapause from the populations in an adjacent (Peter) Lake, which had been connected to Paul Lake *via* a channel until 1951. In mid-November shortly before the lakes become covered with ice, the adults are still reproducing parthenogenetically in Peter Lake. In recent years (1962–64), each lake has contained two species of *Daphnia; D. pulex* is the more abundant in Paul and *D. rosea* is the more abundant in Peter Lake. Neither species enters embryonic diapause in Peter Lake, whereas both do in Paul Lake. Peter Lake is larger than Paul. Its surface area is 2.4 hectares (as compared to 1.2 ha.) and its depth is 17 meters (as compared to 12 m.). The lake has also been modified through the addition of hydrated lime (2500 Kg./ha.). As a result Peter Lake, among other parameters, is optically more transparent and has a smaller biochemical oxygen demand (BOD) (Stross and Hasler, 1960). From the latter one may infer a smaller flux of respiratory gases and other metabolites within the community of organisms.

The autumnal onset of sexual reproduction and diapause in Paul Lake implicates photoperiodic control as readily as some of the controls postulated in the earlier literature. However, the populations of the two species of *Daphnia,* even though they may share a common ancestry with those in Paul Lake fail to respond in Peter Lake. Therefore the supposition may be made that photoperiod is not the stimulus, or that a second stimulus, peculiar to Paul Lake, may act as inducer when photoperiod is of a permissive duration. The same stimulus may be expected to induce sexual reproduction (and diapause) in cultures of *Daphnia* from both lakes. The geometry, *i.e.,* container size and culture density, employed successfully by Berg (1931) to bring about sexual reproduction in cultures of *Daphnia* from a lake with a diapause phenology similar to that of Paul Lake were used in this study to provide some assurance of success in the test of photoperiod.

RESULTS

Photo-sensitive stage

The pre-natal stage is apparently most sensitive to the action of photoperiod. When shifted from long-day (L:D 16:8) to short-day photoperiod (L:D 11:13) as young (0 to 2 days old), the culture produced nearly four times more sexual (ephippial) broods at the short day-length (Fig. 2). Even so, the incidence of sexual broods was considered marginal. Young born at the short-day photoperiod responded intensely, however. A sub-culture begun with young born of parents living in short-day photoperiod for 12 days produced 78.3% sexual broods. A second sub-culture with young born 30 days after transfer produced essentially all ephippial broods. The control cultures of Paul Lake *D. pulex* produced some ephippial broods at long-day photoperiod (Fig. 2). Although normally less than 5%, the incidence rose to as high as 30% (not shown), possibly as a result of the cultures having been fed senescent food (see below).

The presence of sexual reproduction in long days (L:D 16:8) made it difficult to ascertain precisely the requisite number of inductive cycles of light

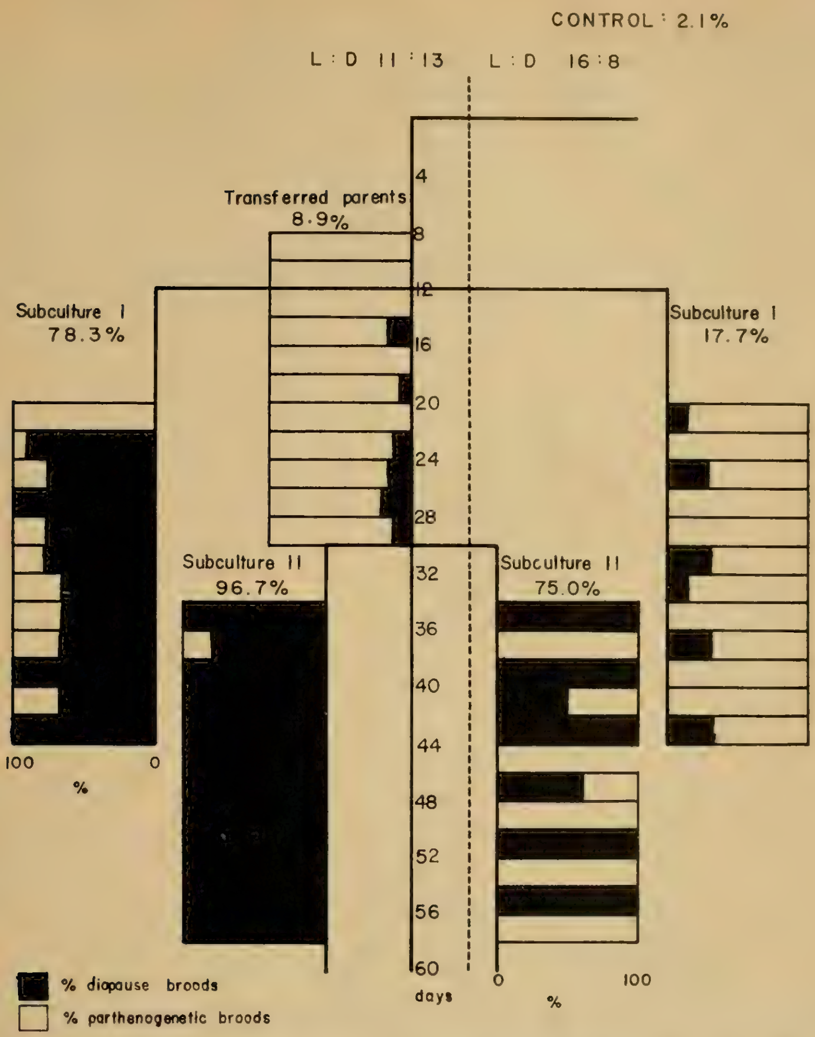

FIGURE 2. Incidence of sexual broods in two successive generations of *D. pulex* (Paul Lake strain). The parents were transferred as young (0–2 days old) from long- (L:D 16:8) to short-day photoperiod (L:D 11:13). Their parthenogenic offspring were started in subcultures on days 12 and 30 of the experiment. Details of culture are to be found in Methods.

and dark. However, slightly more than half of all replicates yielded the first ephippium either on day 16 or 18 following transfer of the *Daphnia* to short days. Reversal from sexual to parthenogenic broods is likewise not complete. Generally speaking, the incidence of diapause on day 4 following transfer from long days was minimal, and the cultures continued throughout life to produce ephippia at a similar level as is exemplified in Figure 2. There is also some indication of a maternal influence on reversal, with young born to older parents less able to reverse.

Critical photoperiod

The switch to sexual reproduction was transitional at 13.5 hours of light at 19° C. (Fig. 3). One-half of the reproduction was in the form of sexual broods at L:D $12\frac{3}{4}:11\frac{1}{4}$. Temperature influenced the critical photoperiod only slightly. At 12° one-half of the broods were sexual at 13:11, a shift of 15 minutes, with a temperature change of seven degrees. Sexual reproduction was maximally intense at L:D 12:12.

The response was not so intense in cultures of the Peter Lake stock when tested at the standard density of 5 individuals/20 ml. Nevertheless, the capacity of cultures from Peter Lake to produce ephippia under short-day photoperiods is demonstrated. In short days (L:D 12:12) at 19° C., 59.3% of the broods were ephippial. No sexual broods were produced at L:D 16:8. At 12° the cultures produced a maximum of 32% sexual broods at L:D 12:12 and 13:11 and none at longer photoperiods. The response of Paul Lake cultures under identical conditions was to produce all sexual broods at L:D 12:12 and 50% sexual broods at 13:11.

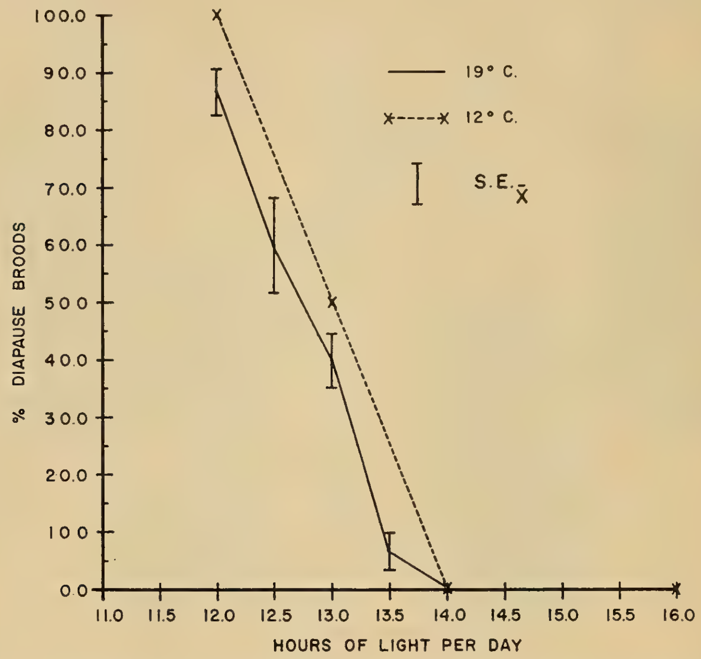

FIGURE 3. Photoperiod and sexual reproduction in cultures of *D. pulex* (Paul Lake strain) started with young born at the indicated photoperiod. A standard density of 5 individuals was maintained in each replicate throughout the experiment (30 days).

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That photoperiodic control is involved was shown by the use of so-called skeleton photoperiods in testing Peter Lake stock. Asymmetric skeletons, consisting of an 8-hour main light period followed after a dark interval by one hour of supplemental light, either permit or suppress sexual reproduction. When the supplemental hour formed a 12-hour day, *i.e.*, 8(3)1(12), sexual broods were produced, but when the supplemental hour formed a 14-hour day, *i.e.*, 8(5)1(10) all broods were parthenogenic.

Second stimulus

The success of skeleton photoperiods makes it possible to rule out an indirect action of light (*e.g.*, photosynthesis of plant cells used as food) as the primary stimulus. Both the field observation and subsequent experiments suggest, however, that a second stimulus is necessary to promote sexual reproduction in *Daphnia*. When cultured at densities other than 5 adults per 20 ml. of medium, the Peter Lake stock was capable under short days, *viz.*, L:D 12:12, of reproducing only sexual or only parthenogenic broods. All broods were parthenogenic at L:D 12:12 at 19° C. when only 3 adults were cultured in 50 ml. of medium, and all broods were sexual at a density of 30 adults per 50 ml. (Fig. 4). Intermediate densities gave intermediate intensities of sexual reproduction. An equal number of cultures with the same densities were transferred from L:D 12:12 to 14:10, and in these no sexual broods were produced after six days at the longer photoperiod.

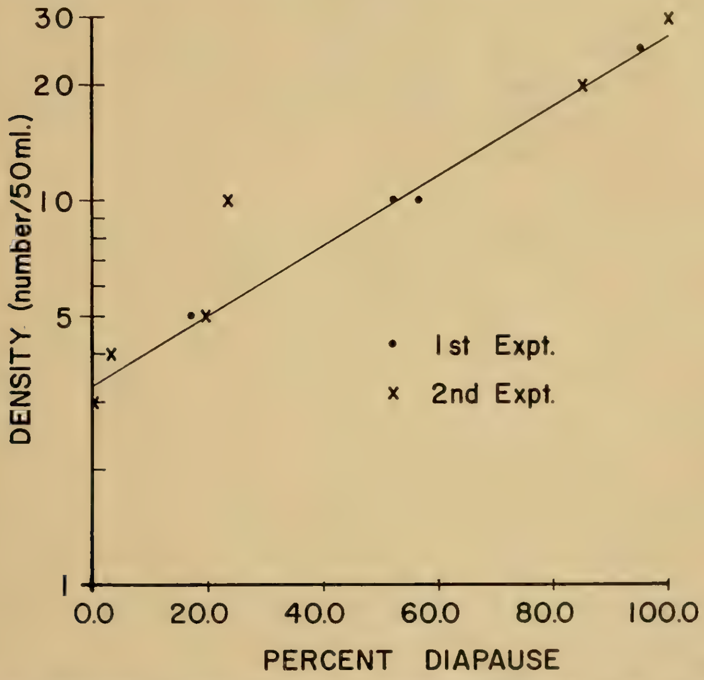

FIGURE 4. Incidence of sexual (diapause) broods of *D. pulex* (Peter Lake strain) at various densities in short-day photoperiod (L:D 12:12). The experiments were started with young that were born at that photoperiod.

The Paul Lake stock of *D. pulex* was also cultured at various densities in short days (L:D 12:12) at 19° but at a different "geometry" of culture conditions. Densities of 1 to 10 adults per 20 ml. of medium produced from 33 to 100% sexual broods (Table I). The shift to sexuality was essentially complete at 5 adults per 20 ml. of medium. All experiments testing the effect of density utilized young that had been born at L:D 12:12.

No attempt was made to substantiate the apparently greater sensitivity of the Paul Lake stocks to crowding, since the experiments were not carried out with clones. One distinguishing feature stands out, however. The Paul Lake stocks retain the potential for exclusively sexual reproduction, as measured by the release of ephippia, after nine months in the laboratory in an inductive photoperiod (L:D 12:12). Stocks from Peter Lake apparently lose the ability to produce ephippia when held in the laboratory for only a few months at either long or short daylengths. Curiously, the Paul Lake stocks fail to produce males after five or seven generations following hatch of the sexual egg in the laboratory, after having demonstrated the ability (see below). There is, therefore, some probability that the populations of the two lakes are genetically different but that the nutrition or other environmental component in the laboratory is partially responsible for the different responses.

TABLE I

Density of adult D. pulex and percentage of sexual broods in a short-day photoperiod (L:D 12:12). Even-age individuals were cultured in 20 ml. of medium (in shell vials). (See methods for culture technique)

Adults/20 ml.	Adult ♀ days	Total broods	Per cent ephippial broods
1	18	6	33.3
2	64	11	72.7
3	86	15	66.7
4	114	24	54.2
5	162	26	96.1
7	202	30	93.3
10	222	31	100.0

The density-related stimulus is suggested to be volatile, at least in part. In a preliminary experiment with Paul Lake stock, the incidence of sexual reproduction was reduced from 32.0% to 0.7% ($P = 0.01$) and from 43.3% to 5.8% ($P = 0.01$) as a result of bi-daily aeration of the cultures. The cultures were maintained at 5 and 10 females/50 ml. of medium, respectively. The medium, which was not changed, was aerated after removing the *Daphnia* to a pipette, by rapid decanting from one beaker to the other. Young were transferred from long day (L:D 16:8) to short daylength (L:D 12:12) for the experiment. A constant amount of food per individual *Daphnia* was supplied every two days. The density-related stimulus may also be simulated by the feeding of cells of *Chlamydomonas* from cultures 23 days old (as opposed to 12). The effect could be indirect, *e.g.*, reduced ratio of photosynthesis to respiration in the older cultures.

Male induction

The appearance of males in broods of normally all-female young was earlier reported to be associated with daylength in the stock from Peter Lake (Stross and Hill, 1965). In a preliminary experiment with the Paul Lake stock, male induction was dependent on daylength, culture density, and apparently on the age of the mother when it was presented with an inductive photoperiod. When born in long days (L:D 15:9) and transferred to short days, the adults produced males consistently only at L:D 12:12 (Table II). Moreover, males appeared in all (4) replicates containing 5 but not 10 individuals per 20 ml. The average frequency was 40.0% with a range of 23 to 83% males. In other combinations males were produced in only one of 4 or 5 replicates, and the average for the treatment did not exceed 4.4%. Curiously, no males were born to adults shifted as young from L:D 15:9 to 14:10. With one exception, no males were produced by females born at a short-day photoperiod (L:D 12:12). Roughly interpreted the results support an hypothesis that male production is synchronized to occur only at the beginning of the shift from parthenogenesis to sexuality and only to adults exposed to non-inductive photoperiods in prenatal life. A second experiment confirmed the hypothesis but further elaboration was not possible since males were not produced in subsequent generations.

Table II

Frequency of male Daphnia pulex (Paul Lake stock) at long- and short-day photoperiods and at two densities. Young for the experiment were collected from parent cultures held at either L:D 15:9 or 12:12 and transferred to the test photoperiod in the first four days after hatching. Each value is a cumulative average of 4 or 5 replicates for days 10 to 30 of the culture. Temperature was 19° + 1.0. (Sexual broods not shown)

	Photoperiod							
	15:9		14:10		13:11		12:12	
	Young	Males (%)	Young	Males (%)	Young	Males (%)	Young	Males (%)
Born at L:D 15:9								
5/tube	70.0	3.6	29.0	0.0	31.2	0.6	17.0	40.0
10/tube	80.8	1.5	63.2	0.0	24.2	3.3	22.6	4.4
Born at L:D 12:12								
5/tube	66.0	0.4	53.8	0.0	32.8	0.0	17.0	0.0
10/tube	24.8	0.0	14.4	0.0	0.0	—	0.0	—

Termination of diapause

Comparative studies have already shown a diversity in the diapause requirements of three populations of *Daphnia pulex*. In the two other than from Paul Lake, one in summer diapause from a small lake and the other from the laboratory, diapause development and release has been shown to be a two-stage process (Stross, 1965).

The first stage of diapause development is refractory and may require an interval of low temperature (*e.g.* 4° C.) for completion. The second stage is light-demanding (*i.e.,* positively photoblastic) and diapause release follows only after an appropriate exposure to white light. Previous reports of winter diapause have shown an extensive refractory period requiring low temperature (Vollmer, 1912; Stross, 1966) but no need of light to permit diapause release. That light influences embryos in winter diapause is indicated through several lines of evidence. When diapause is terminated through a shock treatment, such as with sodium hypochlorite, exposure to light is essential, as it is when embryos which are held in prolonged diapause, through storage in a stagnant medium in constant dark, are transferred to fresh medium at a higher temperature (*e.g.,* 12° and 20° C.). The holding action of a stagnant medium and constant dark is nullified if the embryos are exposed to light. Also, there is some indication, to be explored below, that a short-day photoperiod may delay slightly the termination of diapause in embryos provided the medium is sufficiently well aerated. It is the purpose of the following to describe the influence of light and temperature on the termination of winter diapause in both the laboratory and Paul Lake.

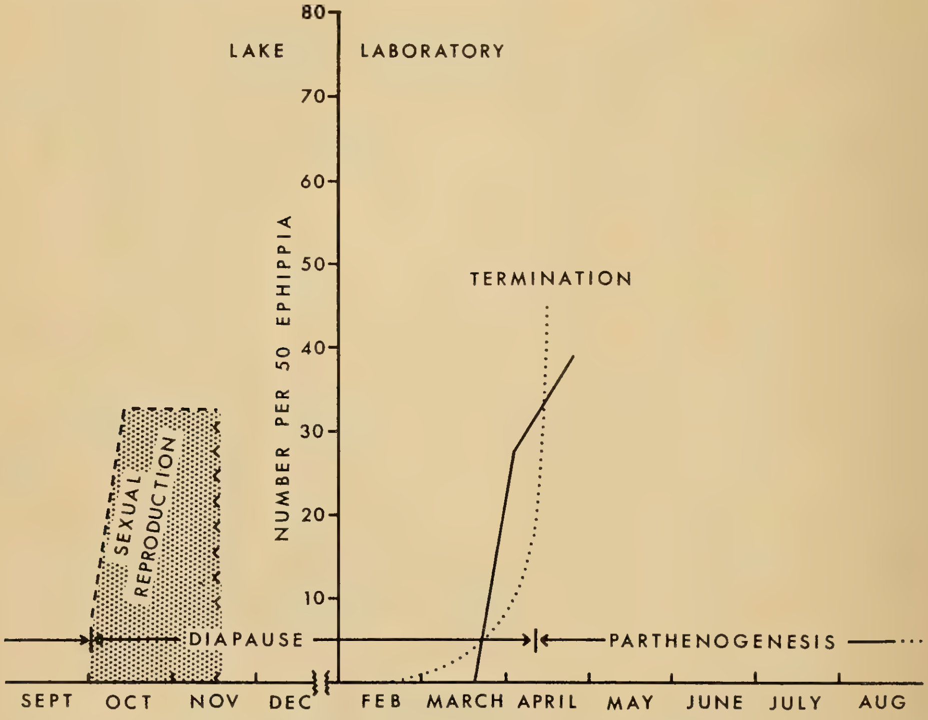

FIGURE 5. Life-cycle of *Daphnia pulex* and (*D. rosea*) in Paul Lake. Sexual reproduction begins in early autumn and the resulting embryos remain in diapause until late March or early April of the year following. Termination of diapause proceeds in constant dark (solid line) and apparently at a somewhat different rate than when the embryos are held in a short-day photoperiod of 11:13 (dotted line). (See text for details.) Hatching of young begins some 3 to 16 days after termination, depending on the temperature.

The embryos are in diapause from inception in October until the following April when post-diapause development begins, nearly 5½ months later (Fig. 5). Diapause development may proceed at 4° but not at 12° or 20° C. All stages up to and including hatching go to completion in the laboratory and Paul Lake. In the laboratory diapause may be prolonged as a result of confinement of the ephippia in floating masses ⅜ to ½ inch thick within sealed containers, provided that constant darkness is maintained. The embryos have been retained in such a manner for an additional four months (August), although decreasing numbers survive the treatment.

Photocontrol of termination is not readily established. Incubation in short-day photoperiods of L:D 11:13 and 13:11 delay termination by an additional two weeks more than incubation in constant dark (Fig. 5). In this experiment the extent of diapause termination was measured by the transfer of embryos from 4° to 20°, thus effectively stopping further diapause development without impairing post-diapause development. That a rise of temperature to 12° or 20° C. blocks further diapause development was deduced by the time interval in which hatching of the

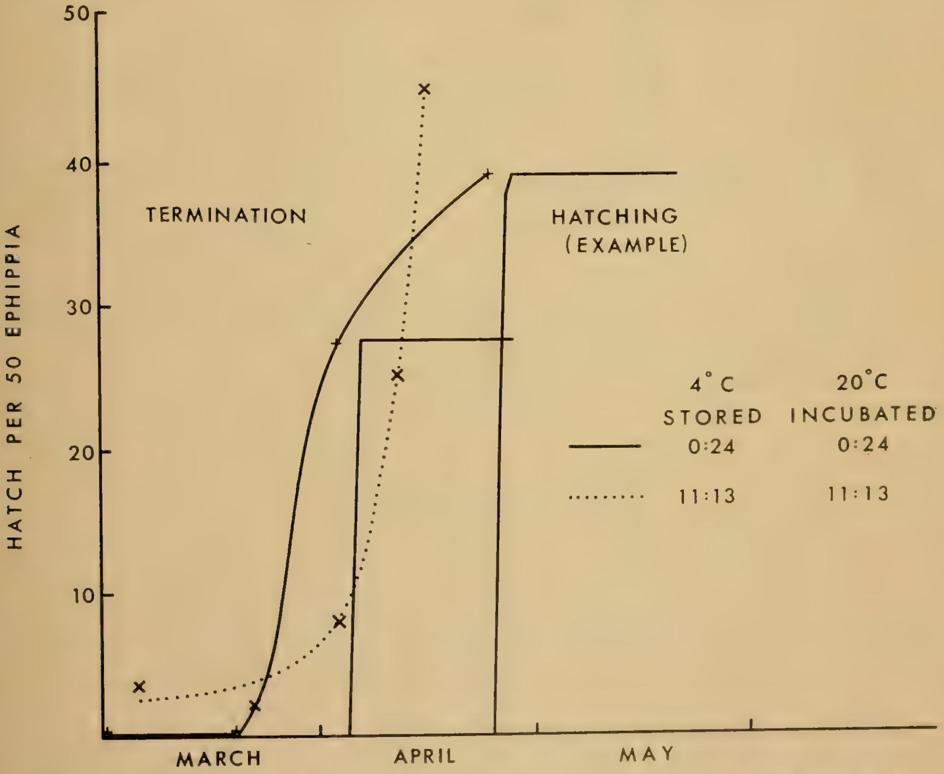

FIGURE 6. Termination of diapause when the embryos of *Daphnia* were held at 4° C. transferred to 20° C. and subjected to either constant dark or a short-day photoperiod of L:D 11:13. The time of termination is estimated from the time of hatching (as shown in part) minus a constant time interval to allow for post-diapause development at the appropriate temperature.

young occurred. None occurred following the predicted length of post-diapause development (Fig. 6). Nor did the transfer to a higher temperature accelerate completion of the refractory period, since a comparable number terminated diapause at the 4° temperature, as deduced when allowance is made for the longer post-diapause interval at the lower temperature (see Methods). The influence of aeration and a procedural manipulation could form a partial explanation for the lack of a clear-cut holding action of short-day photoperiod.

Hatching is delayed in short-day photoperiod (L:D 11:13) as a result of delayed transfer of sub-samples from the stock containers. The reverse is apparently the case when the embryos are held in constant dark (Fig. 7). The interaction of light and aeration is most dramatically illustrated by an observation at a time in April when the rate of termination of diapause in light is maximal (Table III). In the aerated group the fraction that had terminated in the presence of light is only $\frac{1}{3}$ that of ephippia held in constant dark, whereas in the unaerated group the hatch in constant dark is only a fraction of those exposed to light periods

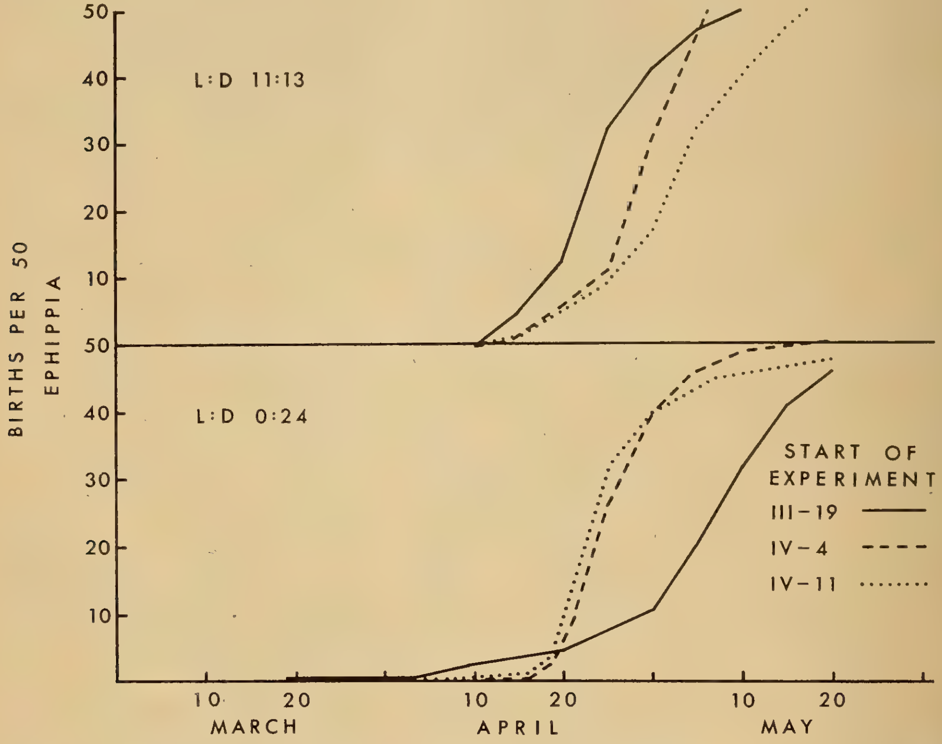

FIGURE 7. Procedural artifact in measuring the time of diapause termination in constant dark as opposed to that in a short-day photoperiod (L:D 11:13). Hatching, as shown by the cumulative hatch, is delayed in short-day photoperiod as a result of delayed transfer of sub-samples from the stock containers. The reverse is apparently the case in constant dark.

TABLE III

Cumulative hatch of Daphnia embryos from Paul Lake, Michigan, on April 19 following incubation at 4° C. since November of previous year. See Methods for details.

Photoperiod	Aerated	Unaerated
L D	$\bar{X}$ ± S.E.	$\bar{X}$ ± S.E.
0–24	26.5 ± 1.5	1.5 ± 0.5
11–13	9.0 ± 1.0	18.0 ± 2.0
13–11	6.5 ± 2.5	35.0 ± 10.0

each day, and presumably would be zero provided the embryos had been maintained in a sufficiently stagnant medium (see above.)

The crux of the problem of establishing photoperiod of diapause termination in embryos from Paul Lake is conceivably procedural. Daily aeration was discontinued when the embryos were transferred from the holding jars to beakers, which would permit the formation of a stagnant micro-zone around the floating embryos. Under such conditions, light could then stimulate rather than suppress termination. A further experiment in which the embryos were suspended in a water column under constant agitation of a stream of bubbles failed as a result of death of the embryos prior to hatching. Unagitated controls survived, however.

The influence of light on diapause termination is much more clearly observed in autumn when, with the appropriate stimuli, some (23%) of the embryos can be forced to hatch. The capacity to respond is apparently transient, since repetition after an additional month of exposure to low (4°) temperature reduced it (Stross,

TABLE IV

Premature termination of embryonic diapause in Daphnia in response to treatment with hypochlorite and incubation in a synthetic medium and light. Each value is total hatch from 2 or 3 replicates with 100 ephippia (approx. 2 embryos) each. The experiment started on November 13 when the ephippia were 2 to 6 weeks old, and continued for one month.

Temp. ° C.	Hypochlorite-treated	Total hatch: Light (L:D 16:8)	Total hatch: Dark (L:D 0:24)
		Lake Water	
20	Yes	1 (2)*	1 (2)
	No	1 (3)	0 (3)
4.5	Yes	0 (2)	0 (2)
	No	1 (3)	0 (3)
		"F" Medium**	
20	Yes	92 (2)	0 (2)
4.5	Yes	0 (2)	0 (2)

* Number of replicates.

** Medium consists of 1000 ml. 2 × distilled water and $NaHCO_3$, 50.0 mg.; K_2HPO_4, 1.0 mg.; $MgSO_4$, 10.0 mg., $CaCl_2$, 80.0 mg.

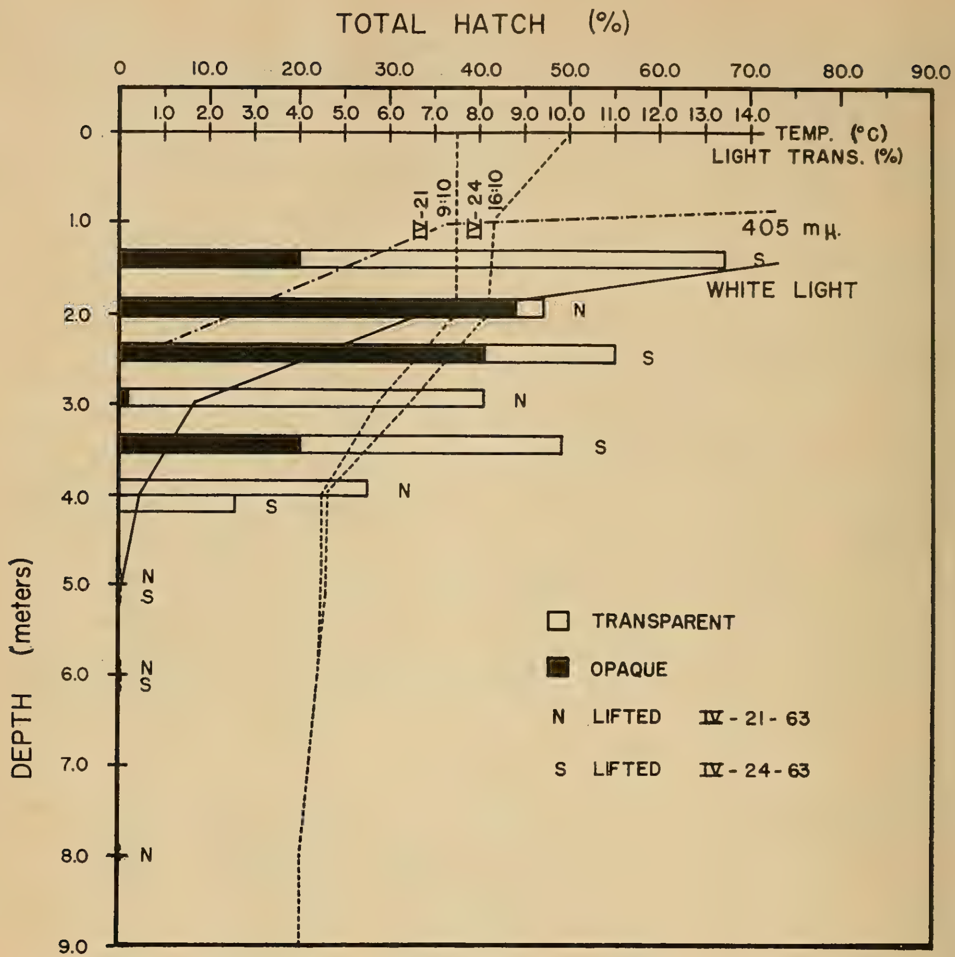

FIGURE 8. Cumulative hatch of *Daphnia* embryos suspended in transparent (white bars) and opaque (black bars) containers at various depths in Paul Lake, Michigan. One series of containers (S) was retrieved 3 days after the other (N). The temperature profile is thought to reveal the extent of wind-driven recirculation, which is incomplete in the lake during the overturn period in Spring. Also shown is the attenuation of white and violet (405 nm) light with depth. The containers were placed in position in November of the year preceding.

1966). In the experiment the embryos were exposed in addition to a long-day photoperiod (L:D 16:8), to a combination of high (20°) and low (4.5°) temperature, a five-minute immersion in a solution of sodium hypochlorite (1.25%), and were incubated in both water from the lake and a synthetic medium (Table IV). Only the embryos exposed to hypochlorite and incubated at the higher temperature in the synthetic medium under light responded in appreciable numbers. Whether or not the synthetic medium is effective without preliminary treatment of the embryos with hypochlorite was not tested.

Termination of diapause in Paul Lake

The results of incubation in the lake may be interpreted from the results obtained in the laboratory. Embryos held throughout the winter in containers suspended in Paul Lake terminated diapause in the natural photoperiod as well as in constant dark. In the first experiment, termination was slightly accelerated in the transparent containers (Fig. 8). The rate of hatching was most rapid in the containers nearest the surface, owing probably to a faster rate of post-diapause development at the warmer temperature. Hatching was restricted to the uppermost 4 meters of the lake which is $\frac{1}{3}$ the total depth. Observations on containers removed from the lake three days later (April 24) substantiated the depth limit while indicating a continued hatch at depths more shallow than 4.0 meters. It was at this depth that a slight temperature discontinuity indicated a marked difference in amount of circulation and presumably in re-aeration of the water after a winter of stagnation beneath an extensive cover of ice and snow. Previous study (unpublished) has disclosed a serious depletion of dissolved oxygen throughout the lake during winter and a persistent anaerobic stratum throughout the spring and summer. It is to be noted also that white light is extinguished to a non-measurable level somewhat deeper than 4.0 meters and that the shorter wavelengths are no longer measurable at a more shallow depth (Fig. 8).

TABLE V

Hatch of Daphnia embryos in the 6 days following removal from Paul Lake on April 21. The embryos had been suspended at various depths in transparent and opaque tubes (Fig. 1a) since November of the previous year and were transferred to beakers for further incubation at 17° C. in natural photoperiod (45° N). Percentage of hatch is based on determination of viable embryos as estimated by dissection of at least 2 groups of 25 ephippia in each container from the lake. (See Figure 8 for hatch in situ.)

Lake container	Transparent		Opaque			
Exposure	Light		Light		Dark	
Number of Ephippia	160		80		80	
Depth (meter)	Hatch		Hatch		Hatch	
	Number	(%) ± S.E.$_x$	Number	(%)	Number	(%)
1.9	0	0.0 ± 0.0	3	1.8	10	6.2
2.9	16	5.0 ± 1.2	109	68.1	100	62.5
3.9	46	14.4 ± 2.5	71	44.4	41	25.6
4.8	210	65.6 ± 2.0	56	35.0	39	24.4
5.8	174	54.4 ± 3.1	48	30.5	21	13.1
7.9	218	68.1 ± 2.5	106	66.2	50	31.2
		Total	393		261	
		Mean		40.9		27.2

Total Hatch: Clear (Lake and subsequent) = 53.7%

Opaque (Lake and subsequent) = 36.4%

The embryos suspended at depths deeper than 4.0 meters proved to be viable and hatched when incubated *in vitro* at 17° C. The number hatching in a six-day interval equalled or exceeded the maximum *in situ* response at the shallow depths (Table V). Embryos exposed to light after having been suspended in opaque cages hatched in consistently larger numbers. Although none of the embryos suspended deeper than 4 meters hatched while suspended in the lake, in subsequent incubation the group at 5 meters hatched well in advance of the requisite interval of post-diapause development, indicating that diapause had been terminated prior to removal from the lake. While the embryos removed from the two deepest strata required the full term (4.0 days), the large response suggested that diapause development had proceeded beyond (phase 1) the point where a rise in temperature (see above) could no longer suppress termination.

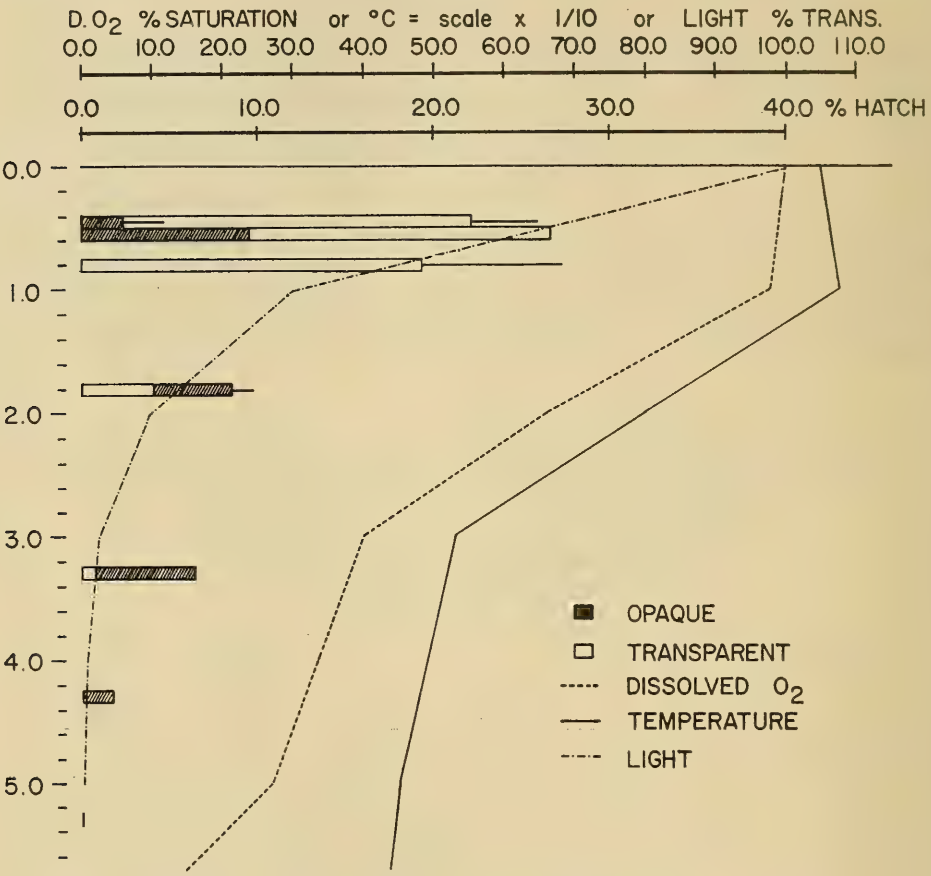

FIGURE 9. Cumulative hatch of *Daphnia* embryos in the experiment of the year following (see Fig. 8 for comparison). The containers were suspended near the center of the lake with the exception of the pair represented by the uppermost histogram. Hatching occurred in all containers. (See Methods for modification of the containers in this experiment).

The pattern of response in the experiment of the following year was quite different, owing presumably to the change in design of containers. The melting of ice was delayed one week to April 21 and the embryos when removed on April 30 from the containers had hatched in fewer numbers than the year preceding. In the containers at depths less than one meter, larger numbers of embryos had hatched in transparent containers and the difference was most pronounced in the groups lying on the sediments of the bottom in the shallows of the lake (Fig. 9). In the cages set deeper than one meter, hatching was more advanced in the opaque tubes. Although the explanation for the radical departure from the results of the year preceding is unknown, the solution could involve the interaction of both light exposure (quality and quantity) and aeration of the embryos. The results of both experiments agree in that the rate of hatching is most rapid nearest the surface of the lake.

Discussion and Conclusions

Alternation of non-sexual with sexual reproduction is common-place in many types of aquatic organisms. Examples such as the water-molds (Emerson, 1954; Emerson and Cantino, 1948), protozoa (Taylor and Strickland, 1938, 1939) hydrozoa (Loomis, 1957, 1959, 1961; Braverman, 1962) and rotifers (Gilbert, 1963) are analogous in that the stimulus responsible for the shift is related to the density of the culture. In several instances a volatile metabolite has been implicated, and in recent studies elevated tensions of CO^2 have been effective (Loomis, 1957, 1961; Braverman, 1962; Emerson and Cantino, 1948), possibly in combination with trace amounts of the Krebs cycle acids in the medium (Loomis, 1964). Other studies have demonstrated the effectiveness of alpha-ketoglutarate as an inducer of sexuality in molds and coelenterates (Braverman, 1962; Chin and Knight, 1957, 1963). A volatile metabolite has been implicated in the induction of male cladocerans other than *Daphnia* (Banta, 1939). The present study is able only to implicate an elevated level of some volatile metabolite resulting from increased density of individuals in culture.

Regardless of the stimulus, the fact is that density-related induction of sexual reproduction (and diapause) in *Daphnia* may require a daylength of permissive duration. Other aquatic organisms may share the capacity to enter sexual reproduction and diapause at short-day photoperiods, if their immediate environment contains the second stimulus. The genus *Hydra* could include such a candidate since in one species (*H. littoralis*) the occurrence of sexual reproduction in nature is mostly during the seasons of short days (Loomis, 1959; Park *et al.*, 1965). Moreover, the experimental protocol and results of Loomis (1959 and elsewhere) are consistent with a photoperiod control in addition to stimulus resulting from CO_2 tension or other metabolites.

The apparent need for two stimuli in an aquatic arthropod is analogous to the many examples from studies of terrestrial insects and mites where temperature (Paris and Jenner, 1959; Danilevskii, 1965) and diet (Lees, 1953; Bull and Adkisson, 1962; Adkisson *et al.*, 1963) may determine expression. However, many of the terrestrial examples result from experimental environments that may be inconsistent with the natural expectation of diet and temperature at the inductive period of the year. Aquatic organisms (at least *Daphnia*) may be unique in that sexual reproduction (and embryonic diapause) may be deferred in the absence of the second stimulus. In the case of *Daphnia* a second, reproductive, type of diapause is possible, since the adult or pre-adult may over-winter in a non-reproductive and motile stage (Brooks, 1965). Whether or not most populations possesses the capacity for both types of diapause is unknown, as is the question of photo-control in the second type of diapause. At any rate the non-expression of diapause in aquatic organisms is obviously possible only if an active stage can survive the winter.

The presence of a motile overwintering stage (and the absence of sexual reproduction) is an indication that the adaptive significance of an embryonic diapause in *Daphnia* may not be based on temperature (Beck, 1963) or available water (Birch and Andrewartha, 1942). Nor does the potential lack of food seem to be a selective force, since it is in the (oligotrophic) lakes with the smallest quantity of suspended food particles that the alternative, motile form of diapause is encountered (Berg, 1931; Brooks, 1964, 1965).

The most serious difficulty that an active stage might encounter is asphyxiation as a result of oxygen depletion (or CO_2 accumulation) under a sealing layer of ice. Complete, or near complete, elimination of dissolved oxygen is known to occur in many lakes (including Paul Lake). In Paul Lake, for example, the deep, perennially cold layer (hypolimnion) contains dissolved oxygen only for a short period following the autumnal circulation of the lake (unpublished), and in summer, oxygen is even depleted in the intermediate layer of sharp thermal gradient (thermocline). In view of the possible presence of the stimulus in Paul Lake during summer, the adaptive significance of a photoperiod-controlled embryonic diapause has two possibilities. One is to insure entrance into the resistant stage prior to a supposed threat of asphyxiation. However, if the second stimulus is present during the long-day photoperiod, it is equally probable that photoperiod control acts to maintain parthenogenesis in the population during spring and summer.

Curiously, the stimuli that bring about structural changes in *Daphnia* preceding embryonic diapause could be the converse of the stimuli that induce cyclomorphosis. Both turbulence (Brooks, 1947) and light (Jacobs, 1962) are necessary for *in vitro* evocation of what is inherently a spring and summer (long-day) phenomenon in certain lakes. Although Jacobs (1962) employed constant light rather than long-day photoperiods, the peculiarity of the response to a limited range of light intensity could indicate that constant light may act as a long-day photoperiod. Hazlewood (1966, and personal communication) has achieved significantly more helmet growth in the long-day photoperiods with and without the provision of turbulence. While the need for light of minimum duration can be understood, the action of turbulence has not been determined. Conceivably, it counteracts the effect of population density in the experimental culture, possibly by physical stimulation of the *Daphnia* thereby overcoming the narcosis of a metabolite (see Banta, 1939, for other kinds of stimulants), or by promoting a greater exchange between medium and atmosphere. Brooks (1964, 1965) has found that cyclomorphic populations over-winter in a "motile" or reproductive diapause, and this observation is consistent with the notion that the absence of the so-called second stimulus in these lakes could permit cyclomorphosis and prevent embryonic diapause, either directly or indirectly as a result of selection for populations incapable of embryonic diapause. In other words the polymorphic expression in *Daphnia* that leads to over-wintering embryos may be short-day inductive and may occur in lakes where community metabolism is intense, whereas the second type of polymorphism (cyclomorphosis) could be long-day inductive and occur in lakes where the community is more dilute and metabolism less intense. This postulate may well serve as a basis for testing the capacity of different populations (and species) for embryonic diapause in a variety of environments.

The termination of diapause in over-wintering embryos is surprisingly synchronous, considering the long duration of diapause development. In the lake, synchrony may be aided by the rising temperature of the surface where the embryos are normally floating. Rising temperatures cannot account for the simultaneity observed in the laboratory where the embryos were maintained in constant conditions. Photoperiod control, or at least some photic influence, cannot be ruled out despite the equivocal results in both lake and laboratory studies. Photoperiod control of termination has been shown for both larval and pupal diapause in insects, both terrestrial (Williams and Adkisson, 1964; Wellso and Adkisson, 1964; McLeod and Beck, 1963) and aquatic (Paris and Jenner, 1959; Englemann and Shappirio, 1965), respectively. Field studies in both habitats indicate an early completion of the refractory phase of diapause development, such that termination is possible long before its actual event in nature (Corbet, 1956; McLeod and Beck, 1963). In the present experiments with *Daphnia* the refractory phase seems to persist until the time of termination, and short-daylength effects only a slight delay relative to events in constant dark. The apparent influence of aeration is to reverse the effect of light and to complicate the experiments. It seems clear, however, that the interaction of light and gaseous environment may promote synchrony of hatching in nature. Together they point out the utility of flotation.

The extreme buoyancy of the embryos (ephippia) may be regarded as a specific adaptation to insure residence in a region of Paul Lake where termination is possible. Another view is to regard flotation as an added mechanism that minimizes the disadvantage of embryonic diapause relative to the reproductive diapause of the adult. In reality the alternative forms of diapause may be in competition within a species. A reproductive diapause should have the advantage in a situation where outcome is predicted by the intrinsic capacity for increase, as is the case in many examples of invertebrates (Andrewartha and Birch, 1954). In synchronous populations oviposition is essentially simultaneous with termination of the reproductive diapause whereas the event occurs nearly a full generation later following termination in the embryo. The delay in first reproduction following termination may have a powerful effect on the intrinsic rate of increase, as illustrated in a general way by Cole (1954). In essence the argument is that embryonic diapause is a resort to a more resistant type of diapause, and its resistance to asphyxiation, for example, as discussed above, makes it preferred to the seemingly reproductively superior and alternative form available to the species. Although possibly coincidental, it may be noted that populations of the dominant herbivore in the boreal oceans, the copepod *Calanus*, are poised during autumn and much of the winter in the last pre-adult instar (Raymont, 1963). Metabolism is only slightly depressed during winter, and the individuals are sufficiently active to maintain their large fat reserves (Marshall and Orr, 1958). Indeed the reproductive diapause in winter seems to be an ideal strategy for synchronizing the maximum birth rate to occur in advance of the large and brief pulse of plant growth during the following spring.

SUMMARY

1. The initiation of sexual reproduction and duration of diapause in the resulting embryo was studied in a population of *Daphnia pulex* Leydig that restricts such to the autumn. Initiation is under the control of a density associated stimulus that is permitted to function only in short-day photoperiods. In short days the proportion of sexual broods is linearly related to the log of culture density.

2. Critical photoperiod (50% sexual broods) at 19° C. was L:D 12 3/4:11 1/4, and only 15 minutes longer at 12° C. The prenatal life of the mother is the more sensitive to photoperiodic induction of sexual reproduction. Exposure to short days after birth may permit a large percentage of parthenogenic broods in which the necessary males are produced, thereby providing males in advance of sexual reproduction in the population. In long days the mothers quickly revert to parthenogenesis, and the extent of reversal is apparently related to age of the grandparent when the mother was born.

3. Sexually derived embryos from a natural population are in diapause from inception in October until the following April when held in the source lake or in the laboratory at 4° C. The embryos complete phase 1 at the end of a refractory period, requiring low temperature for completion. Termination of the diapause follows phase 1, provided that the embryos are in an aerated microenvironment. A limbo, or phase 2 period may be slightly influenced by photoperiod, although termination is only slightly delayed in short daylength as compared to controls in constant dark. The influence of light or short daylength is reversed when the microenvironment is permitted to become stagnant. Under stagnant conditions, phase 2 may persist if the embryos are maintained in constant dark. In other words winter diapause, as exemplified by embryos from Paul Lake, has no obligate need of light for termination, and this is in contrast to the light requirement of other populations of *Daphnia* that have been studied. The exception is noted when diapause is "shock" terminated in autumn shortly after inception.

4. The control of diapause termination and hatching of embryos in the source (Paul) lake are consistent with laboratory observations. Caged in transparent and opaque containers, the embryos terminated diapause at all depths after having spent the winter in position. The rate of hatching was most rapid in cages suspended near the surface, which is where the embryos remain naturally during winter. In the dark diapause terminated more slowly when water exchange was impeded by the container, and more rapidly than in light when water flow was comparable in transparent and opaque cages and when intensity, and probably quality, of light was reduced below the intensity at the surface of the lake.

5. The two-stimulus control of sexual reproduction and diapause in *Daphnia pulex* is examined and analogies between it and terrestrial and other aquatic organisms are described. Also noted is an apparent relationship of the stimuli necessary to induce sexuality and another seasonal polymorphism (cyclomorphosis). Finally, the alternative of embryonic or reproductive diapause is discussed in the context

of a population that must survive the winter and at the same time maximize its intrinsic capacity for increase at the onset of the next period of population increase.

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EFFECTS OF PHOSPHATE CONCENTRATION ON CELL DIVISION RATES AND YIELD OF A TROPICAL OCEANIC DIATOM[1, 2]

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Recently it was suggested that phosphate probably did not limit the rate of growth of phytoplankton in the northeastern tropical Pacific Ocean (Thomas, 1966b). This suggestion was based in part on a comparison of measurements of surface phosphate concentrations in this area with the phosphate concentration found to limit the rate in cultures of a diatom, *Asterionella japonica,* isolated from nearshore California waters. The rate-limiting value for *Asterionella* was 0.25 μg.-at./l. (Goldberg *et al.*, 1951) and surface concentrations in the northeastern tropical Pacific were found to be higher than this value.

However, *Asterionella* may be representative of neritic, temperate populations only, and the rate-limiting value found for this organism may not obtain for tropical, oceanic phytoplankton. Furthermore, Ketchum (1939) had earlier shown that the rate-limiting phosphate concentration for another neritic, temperate diatom, *Phaeodactylum tricornutum*, was somewhat higher than that for *Asterionella*—0.55 μg.-at./l. Thus it was desirable to reinvestigate this problem with an oceanic diatom from a tropical area.

Such an organism has now been brought into axenic culture, and temperature and light requirements for its maximum growth rate have been established (Thomas, 1966a). This organism, *Chaetoceros gracilis,* was isolated from an upwelling area in the northeastern tropical Pacific, the Costa Rica Dome, in 1958.

Limiting nutrient concentrations have been used in formulating various phytoplankton productivity equations—*cf.* Riley (1963) and Steele (1958). These authors have generally assumed a linear relationship between phosphate concentration and the growth rate at concentrations below some limiting value. It might be considered more realistic to use the hyperbolic relationship found by Monod (1942) for specific growth rates and nutrient concentration in bacteria:

$$\mu = \mu_m \frac{(S)}{(K_s + S)} = \frac{1}{x}\frac{dx}{dt}$$

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[2] The authors are grateful to Mr. Edward Renger for performing phosphate analyses of the culture media.

where μ = the specific growth rate, μ_m = the maximum (unlimited) specific growth rate, S = nutrient concentration, K_s =a saturation constant numerically equal to the nutrient concentration at which $\mu = \mu_m/2$ and x is the biomass. The present paper therefore evaluates K_s for phosphate concentration and the diatom *Chaetoceros gracilis,* although the experimental data support the linear relationship used by Riley and Steele. In addition, we present data on the yield of diatom cells as a function of initial phosphate supplied. Both rate and yield results were obtained with batch cultures in which the initial inoculum was about 500 cells/ml. and in which light and temperature were at levels previously found to be optimum.

Materials and Methods

The derivation and description of the diatom culture used in these experiments were previously described (Thomas, 1966a). Axenic stock cultures were maintained in an enriched sea water medium at 21° C. and 500 foot-candles continuous illumination.

The experimental medium was the artificial CF-1 developed by Taylor (1964) except that nitrate and silicate were supplied at 100 μm concentration and phosphate was deleted. Vitamins were also deleted since they had earlier been found to be unnecessary for growth (Thomas, 1966a). This medium contained no chelating agent and was buffered by sodium bicarbonate. The initial pH was 7.6. The medium was prepared in 12-liter amounts and sterilized by filtration through an HA Millipore® filter followed by further filtration through a PH Millipore® filter (pore size 0.30 μ) into a sterile 13-liter Bellco Glass Co. No. 2300 Media Storage and Dispensing Jar fitted with a 47-mm. fritted glass Millipore® filter holder. Following sterile filtration, 500 ml. of the clear medium were dispensed aseptically through the bottom tubulation of the jar into sterile one-liter experimental flasks. Initial HA filtration removed part of the iron from the medium as evidenced by a reddish-brown precipitate on the filter; removal of iron may have affected final yields at high phosphate levels (see Results).

After filtration, analysis of the medium for PO_4-P (Murphy and Riley, 1962) showed that it contained 1.10 μg.-at./l. as a contaminant. Although this was probably enough to support a maximum growth rate, Experiment 1 was set up using this basal medium. Phosphate was added at various levels (the highest concentration was 3.10 μg.-at./l.), and the culture flasks were inoculated at a low level—1 ml. of stock culture per flask. This gave initial cell counts of 180–680 cells/ml. in the various flasks. The flasks were incubated at 27° C. and 800–1100 foot-candles in a glass-bottomed water bath. Light was supplied continuously by cool white fluorescent lamps. Cells were counted at daily intervals with a Coulter electronic cell counter (Maloney *et al.*, 1962; El-Sayed and Lee, 1963).

In Experiment 2, analysis of the basal medium showed that it contained 0.76 μg.-at./l. of PO_4-P. This medium was depleted of phosphate by growing *C. gracilis* in it for 11 days. The medium was dispensed in two-liter amounts into 2800-ml. Fernbach flasks, inoculated with 0.5 ml. of a dense stock culture, and incubated as before. Following depletion, the cells were removed by filtration, additional iron and trace metals were added in the initial concentrations, and the medium was again sterilized by filtration. The phosphate concentration in the depleted medium was 0.07 μg.-at./l. After dispensing the medium, phosphate was added in varying amounts, and the flasks were inoculated with 0.5 ml. of depleted cells grown during the initial depletion. The initial cell count in the various flasks ranged from 252–650 cells/ml. The flasks were incubated and cell numbers were determined daily as in Experiment 1.

In Experiment 3, the initial medium again contained 0.76 μg.-at./l. of phosphate. Depletion was started as before, but after 11 days we found that cell counts were low. Successive addition of iron, trace metals, and vitamins and further incubation did not increase the count.

Finally, we realized that medium CF-1 does not contain added boron and that this batch of medium was prepared in a plastic container. Presumably this nutrient would be supplied by the borosilicate flasks, but our flasks had been in use for some years and the medium could have been boron-deficient. Boron as H_3BO_3 was then added to each depletion flask at a concentration of 2.5 ppm. After 7 further days incubation the cell count had increased 17-fold and the medium was depleted to 0.08 μg.-at./l. of PO_4-P. This observation suggests that *C. gracilis* required boron; a similar requirement in another marine diatom has been shown by J. C. Lewin (1965).

Additional iron and trace metals were then added at the initial level and 2.5 ppm. additional boron was also added; the cells were then filtered off and the medium was sterilized and dispensed as before. Phosphate was added to the various flasks and they were inoculated with 0.5 ml. of phosphate-depleted cells. The initial cell counts ranged from 558–758 cells/ml. The flasks were incubated and growth was determined as before.

Cell division rates were calculated from logarithms of cell numbers at successive times during the exponential phase of growth. The growth rate, μ_2, is the rate calculated using logarithms to the base 2 and its reciprocal is the time taken for the population to double. In this paper the term *growth* rate is synonymous with cell division rate. Growth rates are also calculated as divisions/24 hours.

TABLE I

Growth rates of Chaetoceros gracilis at non-limiting initial concentrations of phosphate

(Experiment 1)

Treatment	PO_4-P concentration (μg. at./l.)	Growth rate	
		μ_2 (hours^{-1})	Divisions/24 hours
A	1.10	0.131	3.14
B	1.12	0.120	2.88
C	1.15	0.132	3.17
D	1.20	0.133	3.19
E	1.25	0.113	2.71
F	1.30	0.137	3.29
G	1.35	0.135	3.24
H	1.60	0.134	3.22
I	1.85	0.134	3.22
J	2.10	0.134	3.22
K	3.10	0.133	3.19

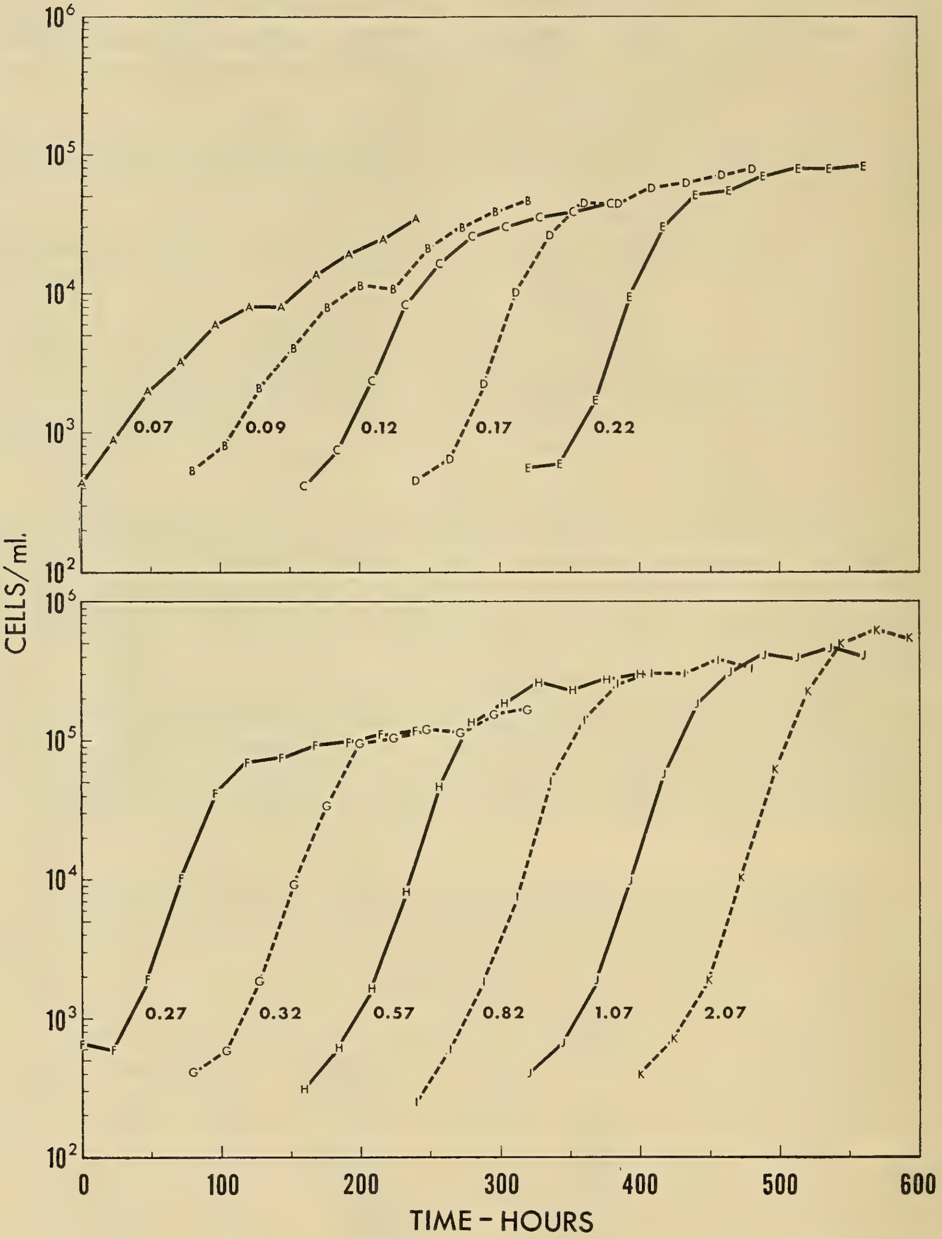

FIGURE 1. Growth of *Chaetoceros gracilis* at several initial concentrations of inorganic phosphate. The growth curves have been displaced 80 hours for clarity. Numbers to the right of each curve are initial phosphate concentrations. *Experiment 2.*

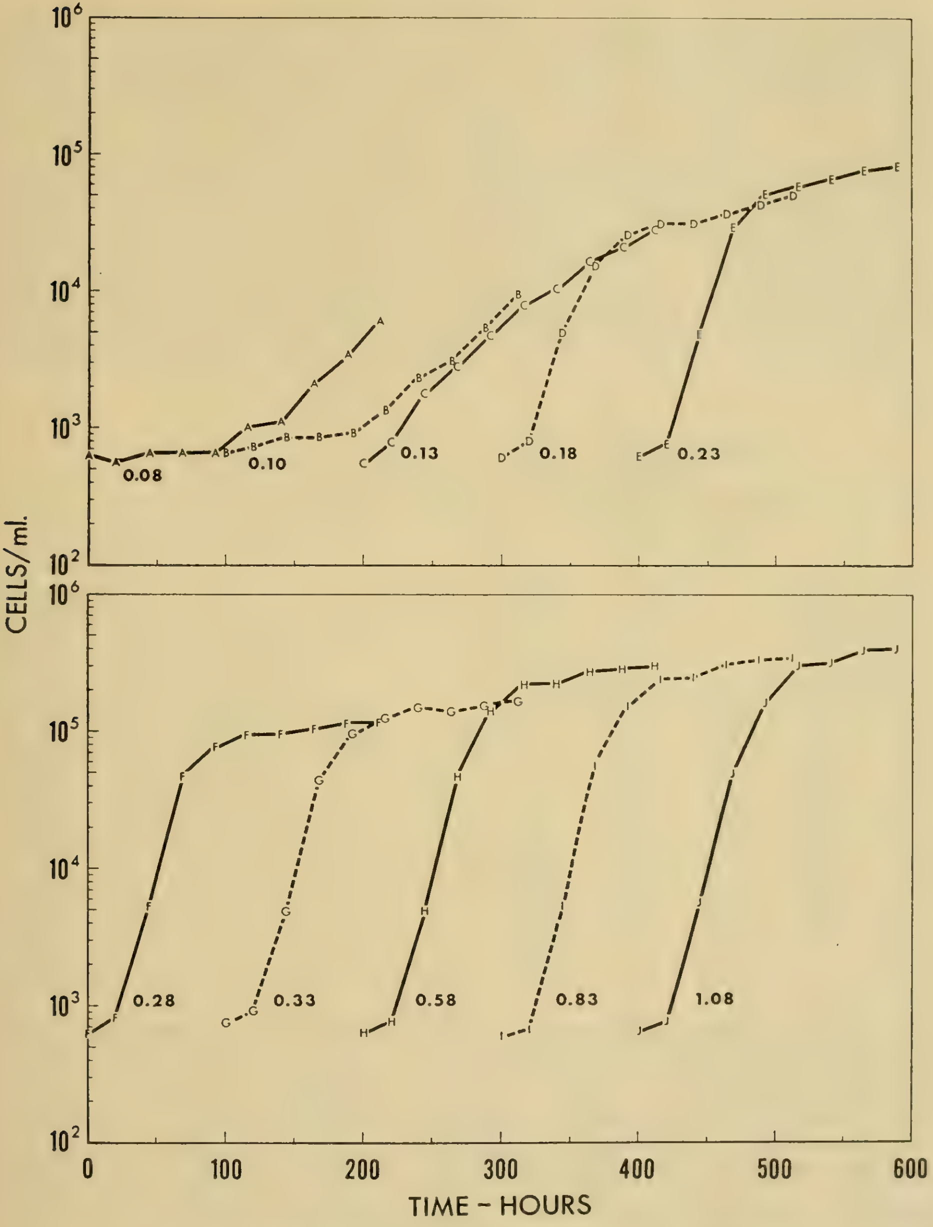

FIGURE 2. Growth of *Chaetoceros gracilis* at several initial concentrations of inorganic phosphate. The growth curves have been displaced 100 hours for clarity. Numbers to the right of each curve are initial phosphate concentrations. *Experiment 3.*

Results

The effects of concentrations of PO$_4$-P greater than 1.10 μg.-at./l. on growth rate are shown in Table I. The rates are all very similar at concentrations ranging up to 3.10 μg.-at./l. and no limitation was found. The rates are about half of the maximum rate found previously (Thomas, 1966a) for *C. gracilis*, but these cultures were not shaken. This experiment was terminated before final cell yields at the higher concentrations were achieved.

Growth curves at low concentrations of phosphate are shown in Figures 1 and 2 in which the curves have been displaced on the time scale for clarity. In Experiment 2 (Fig. 1) there was an appreciable (24-hour) lag period in two treatments (E and F). Otherwise exponential growth occurred almost immediately or the lag period was briefer than 24 hours. At most of the lower phosphate concentrations, growth ceased between 120 and 144 hours and started again thereafter. Probably because of phosphate depletion, some intracellular adjustment in metabolism occurred at this time in these low phosphate cultures, but did not occur in cultures where extracellular phosphate was still abundant.

Table II

Growth rates and final cell yields of Chaetoceros gracilis at limiting and non-limiting initial concentrations of phosphate

Treatment	(Experiment 2)				Treatment	(Experiment 3)			
	PO$_4$-P Conc. (μg. at/l.)	Growth rate		Final yield (cells/ml. $\times 10^3$)		PO$_4$-P Conc. (μg. at/l.)	Growth rate		Final yield (cells/ml. $\times 10^3$)
		μ_2 (hours^{-1})	Divisions/ 24 hours				μ_2 (hours^{-1})	Divisions/ 24 hours	
A	0.07	0.040	0.95	35.6	A	0.08	0.024	0.57	24.4
B	0.09	0.045	1.08	52.6	B	0.10	0.024	0.57	23.2
C	0.12	0.062	1.49	46.0	C	0.13	0.035	0.84	45.2
D	0.17	0.082	1.96	82.4	D	0.18	0.110	2.64	71.8
E	0.22	0.104	2.49	85.4	E	0.23	0.109	2.61	94.0
F	0.27	0.093	2.23	115.0	F	0.28	0.121	2.90	125.2
G	0.32	0.099	2.37	165.4	G	0.33	0.116	2.79	165.4
H	0.57	0.100	2.41	296.0	H	0.58	0.123	2.96	298.0
I	0.82	0.100	2.39	386.0	I	0.83	0.132	3.17	342.0
J	1.07	0.103	2.46	462.0	J	1.08	0.124	2.98	420.0
K	2.07	0.106	2.54	658.0	K	2.08	0.130	3.11	712.0

In Experiment 3, quite long lag periods (92 hours) were found in the two treatments lowest in initial phosphate. Otherwise the lag periods were only 20 hours long. There is a suggestion of cellular adaption to low phosphate (a pause in growth from 116 to 140 hours) in the culture containing the lowest initial phosphate; otherwise the pauses observed in Experiment 2 did not occur.

Growth rates and final yields of cells for Experiments 2 and 3 are given in Table II. The rates increase with phosphate concentration up to a limiting concentration and then remain nearly constant with further increase in phosphate. Final yields increase steadily with increasing concentration. Maximum rates are somewhat larger in Experiment 3 than in Experiment 2. This may be due to an increased boron supply. Rates are still less than those observed previously (Thomas, 1966a) for cultures in enriched sea water medium that also were shaken.

DISCUSSION

Figure 3 expresses the rates for Experiments 2 and 3 as percentages of the maximum observed rate in each experiment. At concentrations above 0.30 μg.-at./l. the rate is 90% of the maximum or greater and is a horizontal function of concentration. Below 0.20 μg.-at./l. the rate decreases sharply and again in a linear manner with decreasing concentration.

The point at which the linear portions of the plot intersect is taken as the limiting concentration and is 0.22 μg.-at./l. Of course, there is some uncertainty

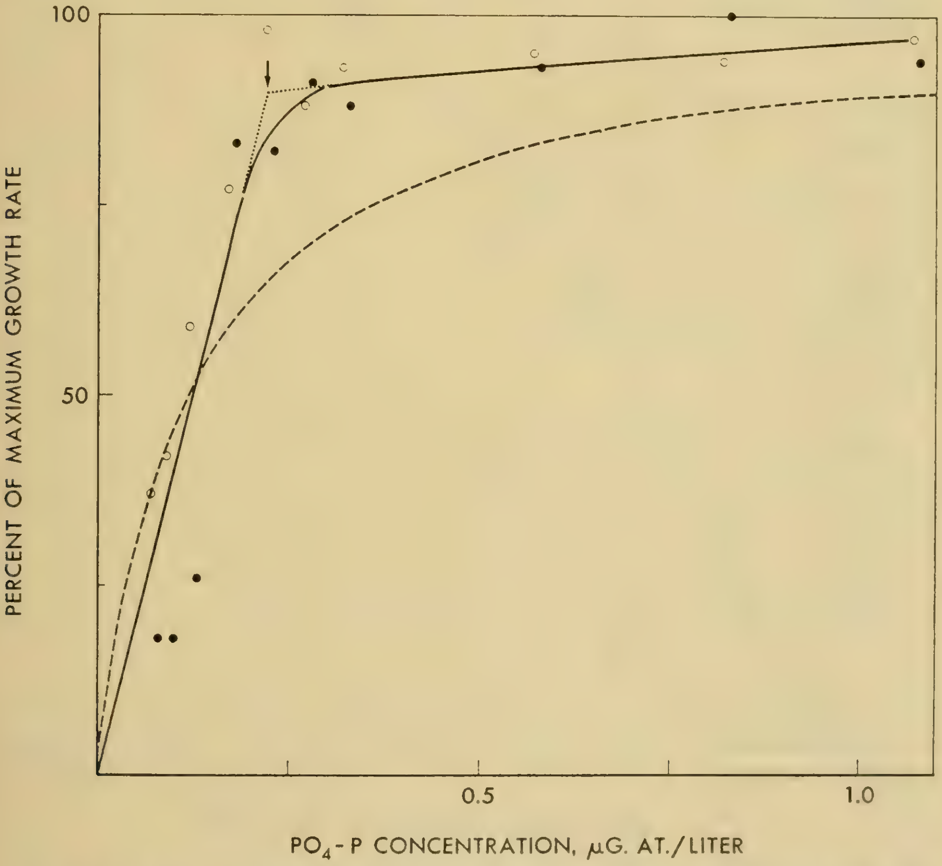

FIGURE 3. Effects of initial inorganic phosphate concentration on exponential growth rates of *Chaetoceros gracilis*. Open circles: Experiment 2; closed circles: Experiment 3. The solid line is fitted to the experimental points and the two linear portions of this curve are extrapolated to the limiting concentration, 0.22 μg.-at./l. The dashed line indicates the curve calculated from Monod's hyperbolic equation using $K_s = 0.12$ μg.-at./l.

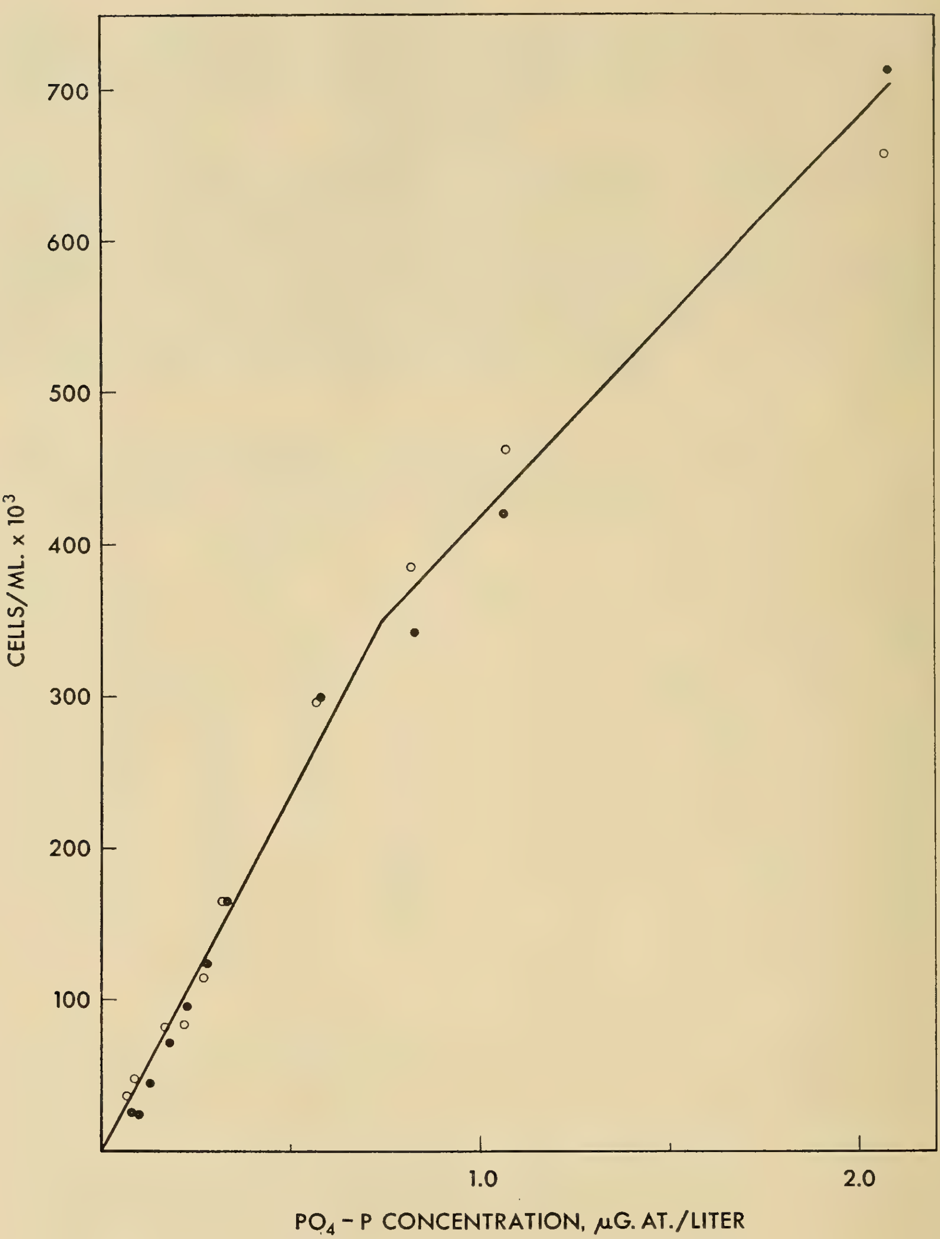

FIGURE 4. Final yield of *Chaetoceros gracilis* as a function of initial phosphate concentration. Open circles: Experiment 2; closed circles: Experiment 3.

about this value and it could range from 0.19 to 0.27 μg.-at./l., depending on how the curve was fitted to the experimental data. This value agrees very well with that found by Goldberg *et al.* (1951) for *Asterionella japonica* and is about half that found by Ketchum (1939). The earlier suggestion (Thomas, 1966b), that phosphate concentrations are not low enough to limit the rate of growth of phytoplankton in the northeastern tropical Pacific Ocean, is substantiated by the present experiments in which an oceanic diatom isolated from this area was used. Mean surface phosphate concentrations range from 0.30 to 0.76 μg.-at./l. in this area and these are greater than the rate-limiting concentration, 0.22 μg.-at./l.

K_s in Monod's equation would be about 0.12 μg.-at./l. However, the experimental points do not fit this hyperbolic equation as is also shown in Figure 3. Rather, there seems to be a linear relationship between concentration and growth rate below the limiting value. Such linear relationships have been used by Riley (1963 and before) and by Steele (1958) in formulating mathematical models of phytoplankton production, and such a relationship appears to be justified by the present data.

Kuenzler and Ketchum (1962) measured the rates of phosphorus uptake by *Phaeodactylum tricornutum* and showed that cells could double their intracellular concentration in about three hours in low-phosphate medium. Since this rate was greater than the growth rate (18 hours doubling time) they suggested (p. 143) that "it seems unlikely that the rate of assimilation of phosphorus would ever limit the rate of growth of a phytoplankton population in nature." While this may be true for the neritic diatom, *Phaedactylum*, it is not necessarily so in the present experiment. The growth rate of *Chaetoceros gracilis* is definitely limited by low phosphate concentrations and either rates of uptake or rates of that metabolism which leads to cell division (or both) are limited. Some enzymatic or uptake sites are not saturated with phosphorus.

The pauses in cell division in Experiment 2 at about 120 hours (see Fig. 1) are probably due to an adjustment in metabolism. It has recently been shown that phosphorus deficiency induces phosphatase enzymes (for example, *cf.*, Fitzgerald and Nelson, 1966) in various algae and in bacteria. Perhaps the pause in growth observed herein is that time taken to induce the necessary enzymes for the utilization of stored phosphorus in the cells but the pause may also be a response to low extracellular phosphate. After this pause, growth continues but at a reduced rate.

The yield of cells as a function of initial phosphate concentration is shown in Figure 4. Final cell numbers are a linear function of initial phosphate up to about 0.8 μg.-at./l. About 500×10^6 cells are formed for each μg.-at. of phosphate; this corresponds to 2×10^{-9} μg-at./cell or 10^{-11} μg.-at./μ^3 of cell material. Goldberg *et al.* (1951) obtained a value for the minimum phosphate per cell of 5×10^{-8} μg.-at./cell for *Asterionella*. Recalculating their results on the basis of the cell volume of *Asterionella*, one obtains about 5×10^{-11} μg.at./μ^3. The present value agrees with Goldberg's within about an order of magnitude, which is perhaps as well as can be expected. At concentrations above 0.8 μg.at./l. the yield decreased somewhat. It seems probable that some other nutrient was limiting; perhaps it was iron, since filtration removed part of the iron from the medium.

SUMMARY

Cell division in batch cultures of the tropical oceanic diatom, *Chaetoceros gracilis*, was measured in artificial medium containing different initial amounts of phosphate. The rate of growth was limited by phosphate concentrations below approximately 0.22 μg.at./l. and appeared to be a linear function of concentration rather than a hyperbolic function. K_s in the hyperbolic relationship equating growth rate and concentration would be about 0.12 μg.-at./l. The results substantiate an earlier suggestion that low phosphate did not limit the rate of phytoplankton growth in the northeastern tropical Pacific Ocean; and they also verify previous use of a linear nutrient relationship in productivity equations given by other authors. Pauses in cell division at low phosphate concentrations are either attributed to the induction of phosphatases which can act on stored intracellular phosphorus or to low extracellular concentrations. Final cell numbers were a linear function of initial phosphate concentration up to about 0.8 μg.-at./l. The amount of phosphorus per cell is 2×10^{-9} μg.-at./cell or 10^{-11} μg.-at./cubic micron. A requirement for boron by this diatom is also suggested by increased growth upon the addition of this nutrient.

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PROTEIN SYNTHESIS BY UNFERTILIZED EGGS OF SEA URCHINS[1]

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In studies of the stimulation of protein synthesis upon fertilization in sea urchins there is some uncertainty concerning the extent to which synthetic activity takes place in the unfertilized eggs. Suspensions of mature unfertilized eggs consistently show some incorporation of amino acid into protein (for references see Monroy, 1965; Monroy and Tyler, 1967; Gross, 1964; Tyler, 1966; Tyler and Tyler, 1966b). However, the nature of this incorporation has been uncertain. There has been uncertainty as to whether it represents messenger RNA-directed protein synthesis rather than an end-labeling of proteins by exchange reactions; and as to whether it may be attributed to the activity of bacteria, or of ovarian tissue, that may be present as contaminants in the preparations. Also, there is the possibility that the incorporating activity may be attributable to only a small percentage of the eggs that had not reached full maturity (although not visibly immature) at the time of shedding.

At the same time it is known that unfertilized eggs possess all the components of a protein-synthesizing system. Thus homogenates of unfertilized eggs can be stimulated by polyuridylic acid, as effectively as can those from fertilized eggs, to incorporate phenylalanine into protein (Tyler, 1962, 1963; Nemer, 1962; Nemer and Bard, 1963; Wilt and Hultin, 1962). Also, the mRNAs for most, or all, of the protein synthesis that occurs during early development are evidently already present in the unfertilized eggs, although they must be largely, if not entirely, in an inactive form, as experiments with non-nucleate fragments (Tyler, 1963, 1966; Brachet, Ficq and Tencer, 1963; Denny and Tyler, 1964) and dactinomycin (Gross and Cousineau, 1963; Gross, 1964) have shown.

That actual protein synthesis may occur in unfertilized eggs of sea urchins is indicated in experiments by Kavanau (1958) who detected a depletion of free amino acids and a concomitant rise in protein after storage for 24 hours. He considered this to represent a continuance of the ripening process.

In order to obtain further information concerning the nature of the apparent protein-synthesizing activity of the unfertilized egg four kinds of experiments were performed. The first was to test the amino acid incorporating ability of supernatant fluids from unfertilized egg suspensions. These fluids would contain bacterial and ovarian tissue contaminants at about the same level as would be present in the egg suspensions. In the second set of experiments the effect of puromycin was tested on amino acid incorporation by unfertilized and fertilized eggs. In the third set polysome-monoribosome "profiles" of unfertilized eggs that had incorporated

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labeled amino acids were examined with respect to location of the label. In the fourth set autoradiographs were prepared of ripe unfertilized eggs that had incorporated labeled amino acids and were compared with those of fertilized eggs. An article by MacKintosh and Bell (1967), that appeared when the manuscript for this paper was written, reports briefly on some experiments similar to those of sets 1, 2, and 4, with results similar to those reported here. Also, a recent paper by Stavy and Gross (1967) reports experiments similar to those of set 3 and in conformity therewith.

Materials and Methods

Eggs of *Lytechinus pictus* were used in these experiments. They were obtained by KCl-injection of the animals, washed and maintained in artificial sea water at pH 8, and given a brief exposure to pH 5 artificial sea water to remove the gelatinous coat as described elsewhere (Tyler and Tyler, 1966a). Measurements of the incorporation of C^{14}-amino acid into protein were made in a scintillation counter (Packard Tricarb) by a modification (Tyler, 1966) of the filter paper procedure of Mans and Novelli (1961).

Experiments and Comments

1. *Tests of supernatant fluids*

The possibility that the incorporation of amino acid into protein by suspensions of unfertilized eggs might be largely, or entirely, due to bacteria and/or contamination by ovarian cells or polar bodies was examined by measurements on supernatant fluids. These were obtained by allowing the eggs to settle after the final washing and removing an egg-free upper portion of the solution. The results of an experiment of this type, that includes also tests with puromycin, are shown in Table I.

Table I

Tests of supernatant sea water from suspension of unfertilized eggs of Lytechinus pictus for incorporation of C^{14}-valine* into protein, in presence and absence of puromycin*

Test material	Solution	Incubation time at 20°C.	Counts per minute	Av.
Unfertilized eggs (11,400)	sea water	0 hours	41 (t$_0$)	43
Unfertilized eggs (11,400)	2×10^{-4} *M* puromycin	0 hours	44 (t$_0$)	
			(minus t$_0$)	
Unfertilized eggs (11,400)	sea water	1 hour	6788,6872	6830
Unfertilized eggs (11,400)	2×10^{-4} *M* puromycin	1 hour	1386,1377	1382
Supernatant sea water	sea water	1 hour	3,10	6.5
Supernatant sea water	2×10^{-4} *M* puromycin	1 hour	−3,−4	−3.5
Supernatant sea water	sea water (no C^{14})	1 hour	3,−5	−1

* A suspension containing 342,000 eggs in 5 ml. was allowed to settle until 2 ml. of egg-free supernatant could be removed. The incubation tubes received 0.10 ml. of the supernatant or of the egg-suspension, 0.15 ml. of the puromycin or of sea water and 0.05 ml. of the C^{14}-valine solution (5 μc./ml. and 208.5 c/M). The supernatant sea water also received 0.10 ml. of the egg-suspensions at the time of quenching, which was done with a 10^5-fold excess of C^{12}-valine at the end of the incubation period. The t$_0$ blanks received the quenching C^{12}-valine at the start.

It is clear from this experiment, and two similar ones that have been run, that the incorporation of amino acid into protein by the supernatant fluid is negligible compared with that by the eggs and, in fact, hardly significantly greater than zero.

It is known from earlier work (Tyler, Ricci and Horowitz, 1938) that suspensions of sea urchin eggs that are in healthy condition contain relatively few bacteria, and this accords with the low amino acid incorporating activity exhibited by the supernatants. There is, however, the possibility that a relatively large number of bacteria might be associated with the eggs themselves. Some experiments (Lonberg-Holm, 1967) on nucleic acid synthesis in lettuce and radish seedlings indicate such an association with bacteria as being responsible for the synthetic activity. Also, in sea urchins there are experiments (Glišin and Glišin, 1964) in which a significant amount of the RNA synthesized by a suspension of fertilized eggs was found to be of bacterial composition. Bacterial counts were therefore made on samples of a supernatant and of the corresponding egg-suspension (lysed). Duplicate sets of serial dilutions of 0.1-ml. samples of the egg-suspension (300,000 eggs per ml.) and of the supernatant after the last washing were plated on nutrient agar (Tyler *et al.,* 1938). After two days' incubation at 37° C. the following figures were obtained for the number of colonies per plate at the 5-fold and the 25-fold dilutions:

Dilution:	5× (egg-susp.)	25× (egg-susp.)	5× (supnt.)	25× (supnt.)
No. of colonies	214;185	47;33	230;191	49;40

From these counts, as well as from the earlier studies, it appears that there are relatively few bacteria (*ca.* 10,000 per ml.) present in the sea urchin egg-suspension as ordinarily prepared, and that there are no greater numbers associated with the eggs than are present in the supernatant fluids.

2. *Sensitivtiy to puromycin*

The inhibiting action of puromycin on the incorporation of C^{14}-valine into protein is illustrated in the experiment listed in Table I. The effect was examined over a range of concentrations in two sets of experiments in which fertilized eggs were tested at the same time. The results are illustrated in Figure 1, where the percentage of the control incorporation which is obtaind at a particular concentration of puromycin is plotted (semilogarithmically) against the concentration.

As the results of these two sets of experiments show, incorporation of the labeled amino acid into protein by the unfertilized eggs is inhibited by puromycin, the degree of inhibition increasing progressively as the concentration of puromycin is increased. Also it appears that the degree of inhibition is of the same order of magnitude as for the fertilized eggs in both experiments, although, in both, the fertilized eggs consistently show greater inhibition at each concentration of puromycin.

The small difference between fertilized and unfertilized eggs may be due to any of a number of factors. For example, there could be differences in permeability to puromycin as well as to the labeled amino acids. There could also be differences in the pool of free amino acids and of the sRNAs at which level inhibition by puromycin is known (Yarmolinsky and De La Haba, 1959; Van der Decken and

Hultin, 1960; Hultin, 1961) to take place. While an understanding of the basis for the difference would be of interest, this would not alter the main conclusion to be drawn from the experiments, which is that the incorporation of amino acid into protein by the unfertilized egg evidently represents protein synthesis.

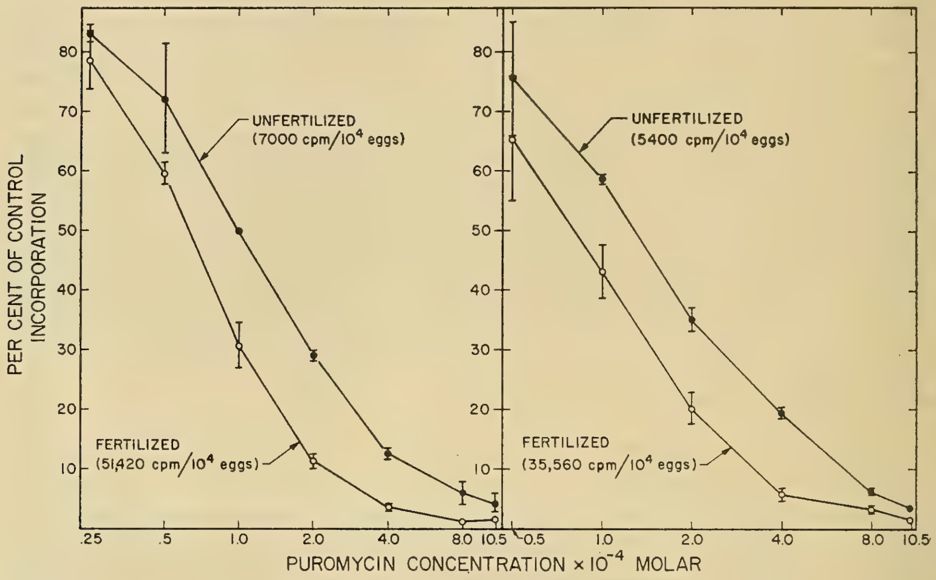

FIGURE 1. Action of puromycin on incorporation of C^{14}-valine into protein by unfertilized and fertilized eggs of *Lytechinus pictus*. The eggs were incubated with the C^{14}-valine for 60 minutes at 20° C., starting 30 minutes after addition of the puromycin, which occurred at 30 minutes after fertilization for the fertilized eggs. Each tube contained in 0.3 ml. final volume, 10,000 unfertilized or 2000 fertilized eggs, puromycin at the indicated concentrations, and 0.25 μc. C^{14}-valine (sp. act. 208.5 μc./μM). Each set of experiments was run in duplicate and the "error bars" are drawn between the two values.

3. *Polysomes in unfertilized eggs*

In several investigations (Monroy and Tyler, 1963; Stafford *et al.*, 1964; Malkin, Gross and Romanoff, 1964; Piatigorsky, 1966; Nemer and Infante, 1967; Stavy and Gross, 1967) it has been noted that the polysome region of sucrose density gradients of homogenates of unfertilized eggs shows RNA-containing material which, however, is largely insensitive to RNase. The low incorporating activity of the unfertilized egg has impeded attempts to determine whether or not some fraction of this material represents polysomes actively engaged in protein synthesis. However, this difficulty has now been largely overcome by the use of non-competing mixtures of labeled amino acids (Tyler, Piatigorsky and Ozaki, 1966).

The results of two sets of experiments with unfertilized and fertilized eggs of *Lytechnius pictus,* labeled with such mixtures, are illustrated in the sucrose density-gradient profiles of Figure 2. In these experiments the eggs were exposed for 20 minutes to the mixture and washed rapidly in cold sea water several times before homogenization. The fertilized eggs were exposed at one hour after insemination, and in each set of experiments unfertilized and fertilized eggs of the same lot were processed at the same time. As the profiles show, both for the unfertilized and the fertilized egg-homogenates, newly synthesized protein, as represented by the incorporated radioactivity, appears primarily in the polysome region of the gradient, aside from that which remains at the top of the gradient. There is some incorporated radioactivity in the monoribosome region, although its specific activity (ratio of cpm to O.D.) is low. The presence of some activity in the monoribosome region is not inconsistent with the general view (Rich, Warner and Goodman, 1963) that polysomes are the primary site of protein-synthesis. It can be interpreted as due to disruption of some polysomes in the preparative procedures and/or to the presence of unseparated protein in the course of normal turnover of polysomes.

The two sets of experiments of Figure 2, and four others that have been run in the present series, show that after RNase-treatment of the homogenates from unfertilized eggs there is a decrease in the incorporated radioactivity in the polysome region of the gradient while a peak of radioactivity appears at a level coinciding with the monoribosome UV-absorbancy peak. In this respect, then, the homogenates from unfertilized eggs resemble those from fertilized eggs illustrated in the same series of experiments and in the earlier experiments to which reference has been made in the introduction. It may then be concluded that true polysomes are present in the unfertilized eggs.

In these experiments the RNase treatment did not result in complete elimination of the UV-absorbing material, nor of the incorporated radioactivity, in the polysome region of the gradient in the homogenates of both fertilized and unfertilized eggs. The former, however, show much greater sensitivity to RNase than the latter. The amount of UV-absorbing material found in the polysome region of the gradient has varied in different experiments and is evidently sensitive to small variations in the conditions of preparation of the homogenates, the initial low speed centrifugation and other factors. Thus, in one of the four additional sets of experiments that have been run, the RNase-treatment of an homogenate of unfertilized eggs resulted in almost complete disappearance of the labeled protein from the polysome region and its appearance in the monoribosome region. In this set there were insignificant amounts of UV-absorbing material in the samples obtained from the polysome region of the gradient. In the other experiments of this series, however, there was RNase-insensitive, UV-absorbing material, associated with nascent protein, in the polysome region of homogenates from both unfertilized and fertilized eggs, although proportionately much less in the latter than in the former, as has been noted in earlier reports cited above.

One possible explanation for the occurrence of these RNase-insensitive, protein-labeled, RNA-containing aggregates, is that the nascent protein itself may keep the ribosomes bound together. This interpretation has been offered by Kretsinger, Manner, Gould and Rich (1964) for the partial RNase-resistance of the collagen-synthesizing, large polysomes from chicken embryos. In this connection, similar density gradient analyses that have been run (Piatigorsky, in preparation) with eggs in which the ribosomal RNA had been labeled (with H^3- or C^{14}-uridine) during oogenesis have consistently shown more complete displacement of the radio-

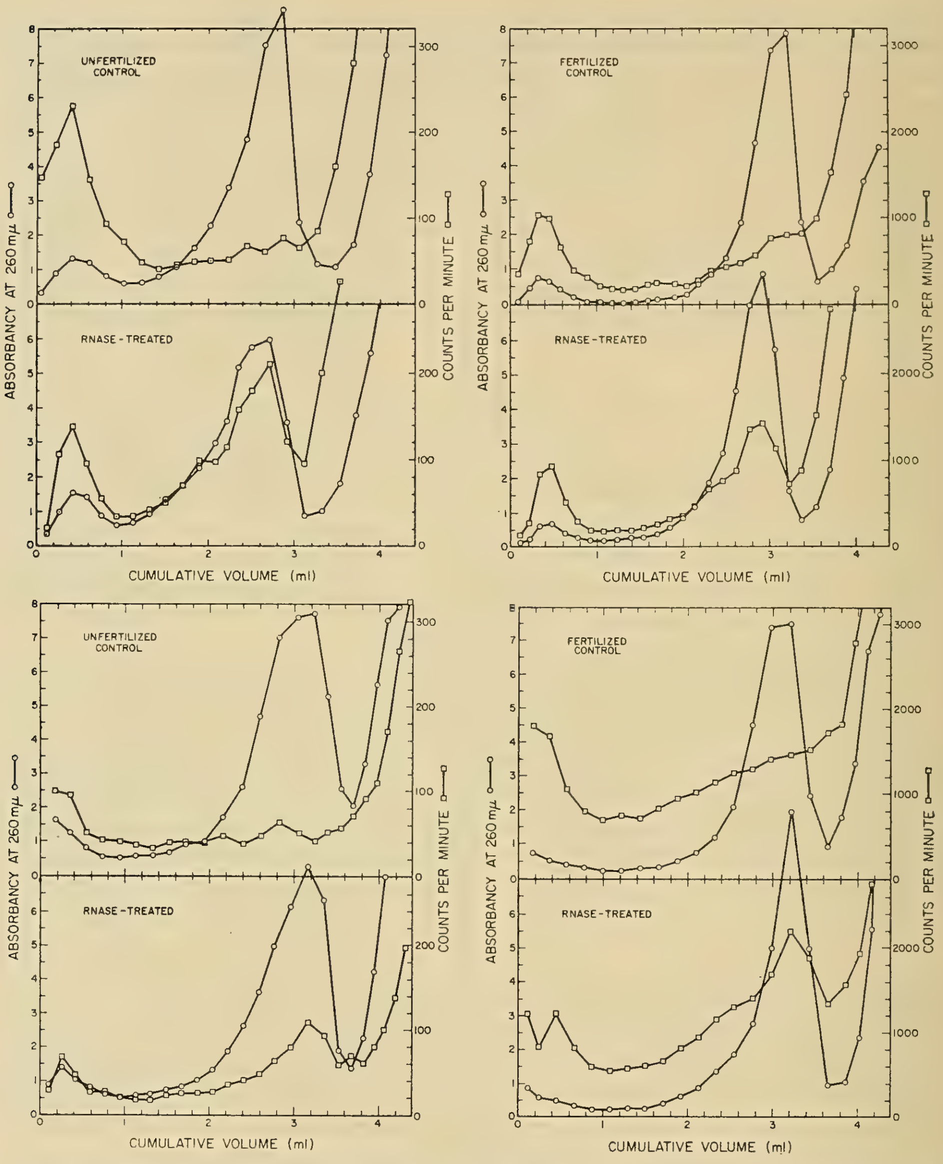

FIGURE 2. Sucrose density gradient centrifugation of homogenates of unfertilized and fertilized (1 hour) eggs of *Lytechinus pictus* following incorporation of C^{14}-amino acids. The two sets of graphs (upper and lower) are for two separate experiments, in which unfertilized and fertilized eggs were processed at the same time. The eggs, in 2 ml. of a 10% suspension, were exposed for 20 minutes to a "non-competing" (cf. Tyler *et al.*, 1966) C^{14}-amino acid mixture (1 μc. each of Val at 195 c/M, Glu at 195 c/M and Arg at 234 c/M), and homogenized in two volumes of buffer (0.025 *M* KCl, 0.004 *M* $MgCl_2$ 0.005 *M* mercaptoethanol), 0.05 *M* Tris, pH 7.4) in a Potter tube with 2 slow strokes of pestle. Removed 0.4 ml. of supernatant after 10 minutes at 10,000 rpm in Spinco SW 39 and divided each into equal parts; added 0.03 ml. RNase (0.01 mg./ml.) to one tube and 0.03 ml. of water to the other; after 1 hour

activity from the polysome region to the monoribosome region of the gradient after RNase-treatment of the homogenates, particularly of the fertilized eggs; also, pretreatment with RNase-free trypsin has sensitized the unfertilized egg aggregates to dissociation by RNase.

4. *Autoradiography of unfertilized eggs*

The presence of labeled protein in unfertilized sea urchin eggs that had been exposed to radioactive amino acids has been demonstrated autoradiographically in previous investigations (Baltus, Quertier, Ficq and Brachet, 1965; Piatigorsky, Ozaki and Tyler, 1967), but the question under consideration here is whether this refers to all of the eggs or only a few. In a recent paper Epel (1967) mentions that an autoradiographic examination of unfertilized sea urchin eggs exposed to H^3-amino acids, showed all to be labeled. Similarly MacKintosh and Bell (1967) state that all examined eggs were labeled in their autoradiographic study. The present results confirm these statements.

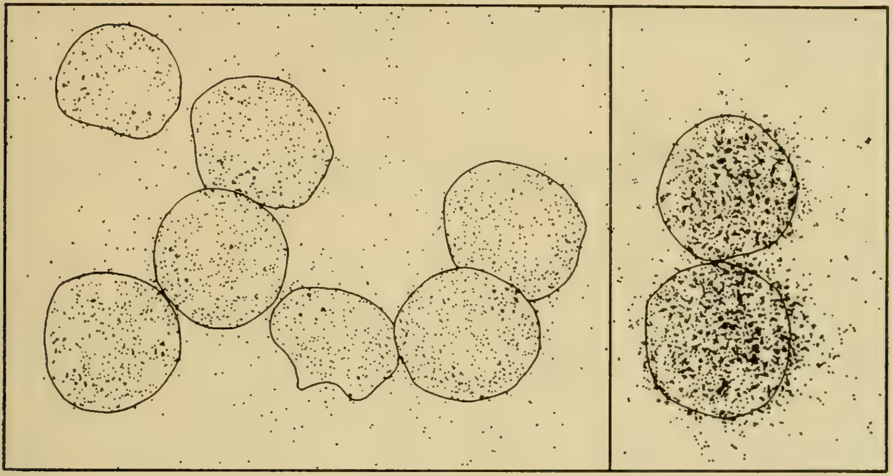

FIGURE 3. Exposed grains on autoradiographs of sections of 7 unfertilized (left) and 2 fertilized (right) eggs of *Lytechinus pictus*. The unfertilized eggs were incubated at 20° C. in sea water containing C^{14}-1-arginine (234 c/M), C^{14}-1-glutamic acid (195 c/M), C^{14}-1-valine (195 c/M) and C^{14}-1-proline (186 c/M), each at 1 μc./ml., for 60 minutes, fixed in Carnoy's solution, sectioned at 10 microns, coated with Kodak NTB3 emulsion, and developed after eight weeks storage at 4° C. The fertilized eggs were incubated in the same C^{14}-amino acid for 30 minutes at 10 minutes after insemination and the emulsion developed after two weeks storage.

Samples of unfertilized and of fertilized eggs of *Lytechinus pictus* that had been

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at 0° C. added 0.03 ml. of 5% sodium deoxycholate to each tube and layered the contents on 15–30% sucrose gradients, with 0.4 ml. cushion of 60%, and centrifuged at 30,000 rpm in the Spinco SW 39 rotor for 100 minutes at 5° C. Three-drop fractions were collected by bottom-puncture of the tubes, the volumes measured and each fraction diluted for measurement of UV-absorbancy (shown as undiluted values on the graphs). The absorbancies (ranging from 0.02 to 0.05) of fractions of a blank sucrose-gradient were also determined and subtracted.

exposed to a mixture of C^{14}-amino acids similar to that used in the experiments of Figure 2, were fixed (Carnoy's fluid), sectioned ($10\,\mu$) and coated with liquid emulsion (Kodak NTB3) for autoradiographic examination. Slides were developed and examined at weekly intervals up to eight weeks. Under the conditions of this test (see legend to Figure 3) the sections of the fertilized eggs showed many exposed grains at the first week, but those from unfertilized eggs were not clearly above background until after the third week. An examination was made of sections of some 50 different eggs on slides that were developed after eight weeks and all showed the presence of many exposed grains. A section of a group of 7 unfertilized eggs of this lot is illustrated in Figure 3 (left) and of two fertilized eggs (Fig. 3 right) on a slide developed after two weeks. The difference in grain-count corresponds approximately to the expectation based on relative incorporation of the labeled amino acids into protein by unfertilized and fertilized eggs.

Discussion

It may be concluded from the results of the four kinds of experiments reported here, and of the similar experiments of others to which reference was made above, that protein synthesis occurs in the ripe unfertilized eggs of sea urchins, that it takes place in association with polysomes as in fertilized eggs and that it occurs to about the same extent in all the eggs rather than in just a few "unripe" ones.

In other species of animals there is evidence for a considerable amount of protein-synthesizing activity on the part of the unfertilized egg. Thus, in starfish eggs Monroy and Tolis (1964) found that the rate of incorporation did not change appreciably upon fertilization, until after extrusion of the second polar body, whereupon it increased rapidly while in the unfertilized egg it declined. However, this type of behavior is not necessarily linked with the stage of the maturation divisions at which the egg is ready for fertilization. In the clam *Spisula solidissina,* whose eggs are also shed in the primary oocyte (germinal vesicle) stage, these investigators found a very rapid increase in incorporation of amino acid into protein very soon after fertilization and well in advance of the formation of the first polar body. MacKintosh and Bell (1967) refer to work by Bell and Reeder (in press) also showing active protein synthesis by unfertilized eggs of this species. According to preliminary reports active incorporation of amino acids by unfertilized eggs also occurs in the polychaete worm *Sabellaria cementarium* (Winesdorfer, 1965) and the gephyrean worm *Urechis caupo* (Gould, 1965). In the frog, *Rana pipiens,* Smith, Ecker and Subtelny (1966) find that full grown oocytes are capable of a low but discrete level of protein synthesis. Upon ovulation, after injection of the donor frogs with a pituitary suspension, and during the ensuing breakdown of the germinal vesicle and progress to the second meiotic metaphase, synthesis increases. Fertilization, or artificial activation, causes no apparent immediate quantitative change.

Evidence that RNAs, too, may be synthesized by unfertilized sea urchin eggs has been obtained by Baltus, Quertier, Ficq and Brachet (1965) and by Siekevitz, Maggio and Catalano (1966). The latter find that the RNAs are of the heterogeneously sedimenting kinds similar to those obtained from fertilized eggs. The low values obtained for this incorporation can, as these workers suggest, be attributed to very low uptake of the uridine precursor (Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966) rather than to low turnover-rate. Apparently almost all of the precursor that does enter is rapidly utilized for the RNA-synthesis.

It appears, then, that the ripe unfertilized egg of sea urchins, and of other animals, is not in a synthetically inactive state. Its activity, however, must represent turnover processes concerned with cell-maintenance inasmuch as the ripe egg may remain unfertilized for a considerable period of time after spawning without evident change in capacity for normal development. The proteins that are being synthesized are presumably not of new kinds but simply represent replacement of those being degraded. During early development of sea urchins, according to the present evidence (*cf.* Spiegel, Ozaki and Tyler, 1965; Terman and Gross, 1966; Tyler, 1966; Ellis, 1966) the soluble proteins that are formed remain for the most part qualitatively the same. Evidence has also been obtained from comparisons of electrophoretic patterns (Ozaki, Piatigorsky and Tyler, 1966) that the soluble proteins synthesized during oogenesis are mostly like those formed after fertilization. Whether or not this may be true also for the proteins synthesized by the ripe unfertilized eggs, is an interesting question for future investigation.

Summary

The known ability of suspensions of unfertilized sea urchin eggs to incorporate amino acids into protein has been examined in four kinds of experiments with *Lytechinus pictus*. These show: (1) that the incorporation is not attributable to bacteria or other contaminating cells in the suspension but to the eggs themselves; (2) that it is inhibitable by puromycin approximately to the same extent as is protein synthesis in the fertilized eggs; (3) that it occurs upon polysomes; (4) that it is a property of all the eggs and not just a small proportion of "unripe" ones in the suspension. It is concluded that the ripe unfertilized egg of sea urchins is not synthetically inert but engages in true protein synthesis although at a much lower rate than during oogenesis or after fertilization.

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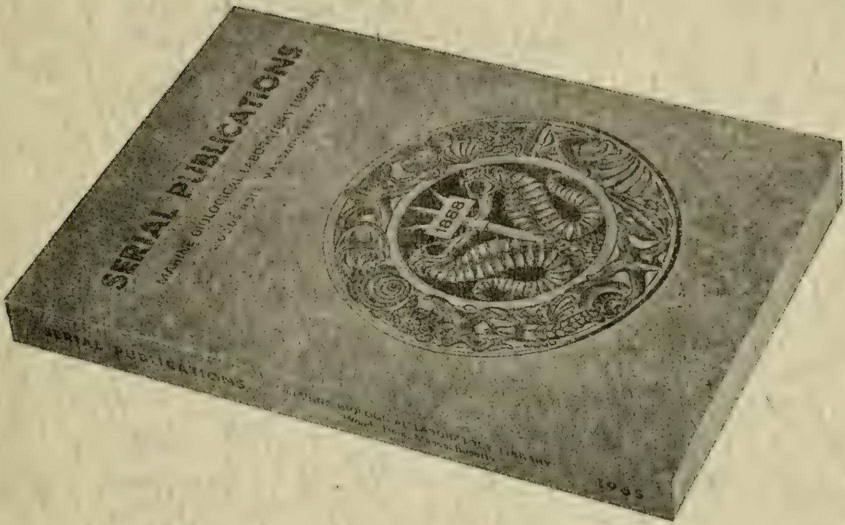
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CONTENTS

Page

D'AGOSTINO, ANTHONY S., AND LUIGI PROVASOLI
Effects of salinity and nutrients on mono- and diaxenic cultures of two strains of Artemia salina 1

ARME, C.
Effects of the plerocercoid larva of a pseudophyllidean cestode, Ligula intestinalis, on the pituitary gland and gonads of its host 15

CAMPBELL, RICHARD D.
Holdfast movement in the hydroid Corymorpha palma: Mechanism of elongation 26

CLARK, MARY E.
Free amino-acid levels in the coelomic fluid and body wall of polychaetes 35

HUGHES, D. A.
Factors controlling emergence of pink shrimp (Penaeus duorarum) from the substrate 48

LENT, CHARLES M.
Air-gaping by the ribbed mussel, Modiolus demissus (Dillwyn): Effects and adaptive significance 60

LOFTS, BRIAN, GRACE E. PICKFORD AND JAMES W. ATZ
The effects of low temperature, and cortisol, on testicular regression in the hypophysectomized cryprinodont fish, Fundulus heteroclitus 74

MACHIN, JOHN
The permeability of the epiphragm of terrestrial snails to water vapor 87

MCALISTER, ROBERT O., AND FRANK M. FISHER
Responses of the false limpet, Siphonaria pectinata Linnaeus (Gastropoda, Pulmonata) to osmotic stress 96

OGLESBY, LARRY C.
Responses of an estuarine population of the polychaete Nereis limnicola to osmotic stress 118

OLSEN, DAVID
Banding patterns of Haliotis rufescens as indicators of botanical and animal succession 139

PATTON, WENDELL K.
Feeding habits, behavior and host specificity of Caprella grahami, an amphipod commensal with the starfish Asterias forbesi 148

SLÁMA, K., M. SUCHÝ AND F. ŠORM
Natural and synthetic materials with insect hormone activity. 3. Juvenile hormone activity of derivatives of p-(1,5-dimethyl-hexyl) benzoic acid 154

STEVENSON, J. ROSS, RICHARD H. GUCKERT AND JANIS D. COHEN
Lack of correlation of some proecdysial growth and developmental processes in the crayfish 160

STROSS, R. G., AND J. C. HILL
Photoperiod control of winter diapause in the fresh-water crustacean, Daphnia 176

THOMAS, WILLIAM H., AND ANNE N. DODSON
Effects of phosphate concentration on cell division rates and yield of a tropical oceanic diatom 199

TYLER, ALBERT, BETTY S. TYLER AND JORAM PIATIGORSKY
Protein synthesis by unfertilized eggs of sea urchins 209

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COMPARATIVE ASPECTS OF THE CHROMATOPHORIC RESPONSES TO LIGHT AND TEMPERATURE IN FIDDLER CRABS OF THE GENUS UCA

FRANKLIN H. BARNWELL[1]

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Fiddler crabs undergo a variety of physiological color changes in their natural habitat (Crane, 1944). These alterations in shade and hue are produced by the concentration and dispersion of pigments within a well developed system of chromatophores. Although several aspects of color change in fiddler crabs have been examined in detail, few experimental studies have dealt with its biological significance. In only one species, *Uca pugilator* (Bosc), has there been a systematic investigation of the chromatophoric responses to light intensity, shade of background, and temperature with a discussion of their possible functional roles (Brown and Sandeen, 1948). In view of the extensive adaptive radiation and the diversity of coloration within the genus (Crane, 1941), it is of interest to learn to what extent the findings with *Uca pugilator* may apply to other species. In this paper some chromatophoric responses to light and temperature are described in two tropical species from Costa Rica, *Uca galapagensis herradurensis* (Bott) and *Uca zacae* (Crane), and a temperate species, *Uca pugnax* (Smith). The results are then compared with the earlier findings on *Uca pugilator*.

MATERIALS AND METHODS

The Costa Rican crabs were collected in March and April, 1966, on the Pacific coast at the Quebrada Salina at Mata Limón, Puntarenas Province. The geographic co-ordinates of Mata Limón are 9° 56′ N lat., 84° 43′ W long. The crabs were taken inland to San José and were used in experiments within one or two days of collection, except where noted.

The specimens of *U. g. herradurensis*[2] inhabited the upper reaches of an open

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[2] Although the crabs used in these experiments have been referred to *Uca galapagensis herradurensis* (Bott), there is a strong possibility that this form should be synonomized with *Uca macrodactyla* (Milne-Edwards & Lucas). Bott (1954) characterized *U. g. herradurensis*

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tidal flat of dark mud which had been cleared of mangroves. The region of the crabs' burrowing extended from above HWNT to HWST, where it was bounded by a steep railroad embankment. During one of the collections, on March 25, the males were observed to be displaying, but no egg-laden females were collected at any time.

The specimens of *U. zacae* were collected on April 16 in partly shaded, sandy mud between HWNT and HWST. The males were displaying at the time of collection. This is one of the smallest species of *Uca;* the crabs used in the experiments, all males, were from 3.9 to 5.7 mm. in carapace length. The species has been taken on the Pacific coasts of Nicaragua and Costa Rica (Crane, 1941).

Collections of *U. pugnax* were made in August, 1966, at the Chapoquoit and Sippewissit marshes on Buzzards Bay near Woods Hole, Massachusetts. *U. pugnax* occurs on the Atlantic coast from Massachusetts to central Florida. The specimens of *U. pugilator* studied by Brown and Sandeen (1948) were also collected in the vicinity of Woods Hole. *U. pugilator* is distributed from Massachusetts to the Florida Keys and has been reported from Haiti (Rathbun, 1917).

The degree of dispersion of the pigment within the chromatophores was determined with a stereomicroscope according to the scale of Hogben and Slome (1931). Stage 1 of this scale signifies maximum concentration of pigment and stage 5, maximum dispersion, with stages 2, 3, and 4 representing intermediate degrees of dispersion. The melanophores and leucophores of a restricted portion of the anterior (ventral) surface of the merus of the third or fourth walking leg were studied. In a few experiments indices of pigment dispersion in the erythrophores of *U. g. herradurensis* were obtained. The erythrophores could not be indexed readily at all times because they were masked by the dispersion of pigment in the melanophores.

It appeared from cursory examination that the melanophores were distributed over the entire anterior surface of the merus in all specimens. By contrast, the leucophores were not so uniform in their distribution. A quantitative analysis of the distribution of chromatophores was not undertaken, but a brief qualitative description is given here. In all specimens of *U. zacae* the leucophores were concentrated on the antero-distal portion of the merus and along the carpal joint. In *U. g. herradurensis* the distribution of leucophores appeared to be correlated with the size of the crab. In the smallest crabs the leucophores of the anterior face of the merus were restricted to its distal portion, as in *U. zacae*. In the remaining specimens the leucophores were present over the entire anterior surface, although

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as a large crab (male carapace length, 12 mm.) with a long-fingered major cheliped, and he accepted Rathbun's (1917) description of *U. macrodactyla* as a small crab (male carapace length, 8 mm.) with a short-fingered major cheliped. However, Crane (1941) reported the occurrence of both long- and short-fingered males of *U. macrodactyla* and she recorded a carapace length of 11 mm. for a male of this species. In the present collection the largest males (maximum carapace length, 13.5 mm.) clearly fit Bott's description of *U. g. herradurensis* and some of the smaller crabs conform with Rathbun's description of *U. macrodactyla*. In view of the possible identity of the two forms, it should be noted that *U. g. herradurensis* has been reported previously only from El Salvador (Bott, 1954; Peters, 1955), whereas *U. macrodactyla* is distributed over 61° of latitude, from Guaymas, Mexico (28° N) to Valparaiso, Chile, (33° S). Specimens of both *U. g. herradurensis* and *U. zacae* from these experiments have been placed in the collection of the U. S. National Museum.

their concentration in the antero-distal region remained obvious. This concentration of leucophores was evident in crabs under natural conditions on the tidal flat as a gray fringe on the upper edge of the otherwise darkly pigmented walking leg. Furthermore, in the laboratory both leucophores and melanophores in proximity to the carpal joint were sometimes the first on the merus to respond to certain experimental conditions, especially at night.

Because of the differential distribution and response, the chromatophores were always staged in the same region of the merus. At the time of staging, the anterior face of the merus was divided visually into thirds, running longitudinally from the upper to the lower edge. An index number of the Hogben and Slome scale was assigned to the chromatophores in the central third of the leg, except for the specimens of *U. zacae* and the young of *U. g. herradurensis* in which the leucophores were confined mainly to the distal third of the leg.

For purposes of comparison the procedures adopted for this study were similar to those used by Brown and Sandeen (1948) with *U. pugilator*. Different backgrounds were provided by putting the crabs in pans painted either glossy white or mat black. The white pan reflected 54% of the incident light and the black pan, 1/30th of this amount.

The effect of light intensity on the chromatophores of *U. g. herradurensis* was examined by placing four pairs of black and white pans, containing ten crabs each, at different distances from a 300-watt incandescent light bulb. The light intensities at the level of the crabs were estimated with a Weston exposure meter to be 120, 32, 12, and 5 foot-candles. After one hour the chromatophores of the crabs in each pan were staged, and the positions of the pans were changed. This procedure was repeated hourly until each pan of crabs had been exposed to each of the four light intensities. The four values obtained for each intensity were then averaged. The experiment was carried out twice during the day between 0900 and 1400 CST and twice during the night between 2100 and 0200. Room temperature during the day was about 23° C. and at night, 22°.

Of the 160 crabs collected for the first daytime and nighttime experiments, 31 proved to be of a different species and were excluded from further consideration.

For examining the pigmentary response to temperature in the two tropical species, ten crabs were placed in glossy white and mat black metal pans in a depth of sea water just sufficient to cover their carapaces. The pans were set in a water bath where the light intensity was 32 foot-candles. Each sample of crabs was exposed sequentially to six temperatures by raising the temperature of the water bath from 15° to 40° C. in steps of 5°. At each level the temperature of the water in the pans was held constant for one hour with a maximum variation of 1°. After an hour at each test temperature the chromatophores were indexed. The experiment was carried out at least twice with each species between 0800 and 1600 CST.

The same experiment was performed twice with *U. pugnax* at Woods Hole, Massachusetts, except that the temperature range was from 10° to 35° C. Light intensity was 35 foot-candles. In two additional experiments the exposure to temperature was started at 35° C. and dropped to 10° C. in steps of 5°. The experiments were all performed during the daytime between 0820 and 1615 EDT.

RESULTS

Effect of light intensity on the response to background

Uca galapagensis herradurensis

Because the results of the first experiment were reproduced closely with a second collection of crabs, the results of both experiments have been averaged in Figure 1.

In considering first the melanophores (Fig. 1A), it is evident that a daily rhythm influenced the magnitude of the response to background. During the day the black pigment was about equally dispersed on black and white backgrounds. At night the black pigment concentrated, but obviously more on the white background than on the black one. In response to increasing light intensity the pigment tended to disperse in the melanophores, as well as in the erythrophores (Fig. 1B) and the leucophores (Fig. 1C).

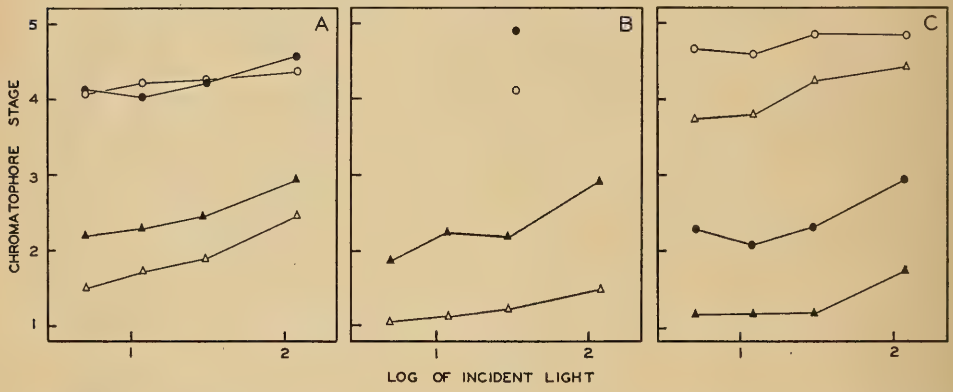

FIGURE 1. Relationships between pigment dispersion in (A) melanophores, (B) erythrophores and (C) leucophores of *U. g. herradurensis* and the logarithm of the incident light intensity. Open circles, white background during day; closed circles, black background during day; open triangles, white background at night; closed triangles, black background at night.

The erythrophores (Fig. 1B) during the night exhibited responses to light intensity and background resembling those of the melanophores. The red pigment was more dispersed on the black background than on the white one. Included in Figure 1B are two values, each obtained from ten crabs in one of the later daytime temperature experiments (temperature, 25° C.; light intensity, 32 foot-candles). These points, along with those obtained at night, demonstrate that the response of the erythrophores to background was superimposed on a daily rhythm in which pigment on both backgrounds was more dispersed during the day than at night.

The leucophores reacted strongly to background with dispersion of white pigment on the white background and concentration on the black one (Fig. 1C). The response of the white pigment to background was much stronger, and the influence of the daily rhythm much less, than in the melanophores.

The chromatophoric response to background, evident in Figure 1, can be resolved into two categories (Brown and Sandeen, 1948). The "primary" response typically involves dispersion of black and white pigments with increasing total illumination. The "secondary" response is a specific reaction to the albedo of the background, defined as the ratio of the reflected to the incident light. The albedo response of the black pigment is generally that of concentration on a white background (relatively high albedo) and dispersion on a black background (relatively low albedo). The white pigment reacts in the opposite way. The primary response at a given light intensity would produce greater dispersion of both black and white pigments on the white background than on the black one, since the white background in these experiments reflected 30 times more light onto the chromatophores of the ventral merus than did the black background. The primary response to the white background would enhance the secondary response in the leucophores but antagonize it in the melanophores.

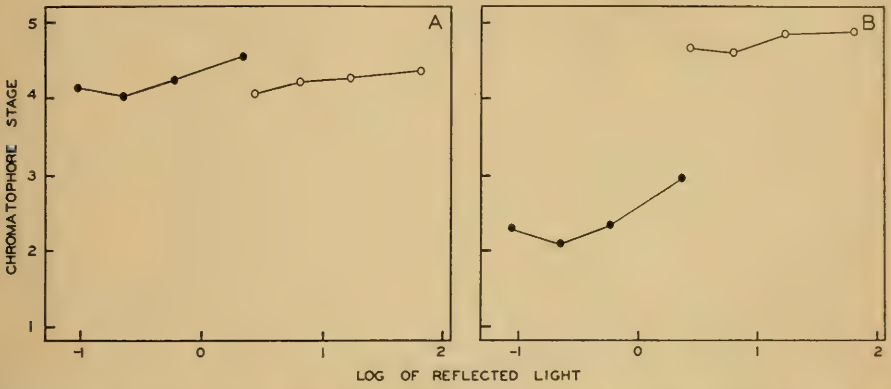

FIGURE 2. Relationships between pigment dispersion in (A) melanophores and (B) leucophores of *U. g. herradurensis* and the logarithm of the reflected light intensity during the day. Open circles, white background; closed circles, black background.

That both types of response are operating is demonstrated in Figure 2 where stage of dispersion of the black and white pigments during the daytime is plotted against the logarithm of the reflected light intensity. The range of reflected light intensities for the black and white backgrounds do not quite overlap. However, at the point of near overlap in both melanophores and leucophores there is a clear shift in level between the responses of the crabs on black and white backgrounds. By a slight extrapolation it is evident that for the same intensity of reflected light, the black pigment is more dispersed on the black background than on the white one. For the white pigment the dispersion is greatest on the white background. Both displacements are in the direction expected if the secondary, or albedo, response was operating. That the primary response was also present has already been indicated by the increased dispersion of pigment with increased light intensity on a constant background.

Effect of temperature on the response to background

Uca galapagensis herradurensis

The chromatophoric responses of *U. g. herradurensis* to temperature are represented by three sets of curves (Fig. 3) which differ primarily in the degree of concentration of the white pigment on a black background.

In the daytime experiment with light intensity the white pigment was observed to concentrate strongly on a black background (Fig. 1C). Crabs from the experiments on light intensity were used four and five days later in two temperature experiments. The results in both temperature experiments were similar and have been averaged in Figure 3C. From the figure it is clear that the white pigment did not concentrate on the black background to the extent that it had under comparable conditions in the experiment with light intensity. Because of the failure to confirm the earlier results, the temperature experiment was repeated with two different groups of crabs on the first day following their collection. The average results of the two experiments are presented in Figure 3A. It can be seen that the white pigment concentrated strongly on the black background, as it had in the previous experiment with light intensity. Crabs from one of the collections were then held in the laboratory in natural light on a black background, as had been the crabs in Figure 3C, and tested again on the sixth day after collection. The responses of the crabs to this experimental treatment have been presented separately (Fig. 3B), because the degree of concentration of the white pigment was intermediate between that in Figure 3A and Figure 3C. These results suggest that the response of the leucophores depended upon whether or not the crabs had been held for many days in the laboratory prior to the experiment.

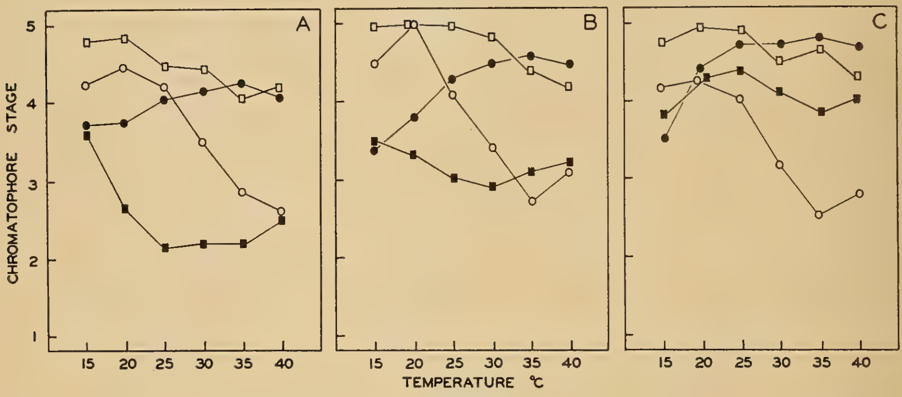

FIGURE 3. Relationships between pigment dispersion and temperature in specimens of *U. g. herradurensis* which were tested (A) on the first day after collection, (B) on the sixth day after collection and (C) on the fifth and sixth days after collection. Open circles, melanophores on white background; closed circles, melanophores on black background; open blocks, leucophores on white background; closed blocks, leucophores on black background.

The three sets of curves in Figure 3 are similar in a number of ways. However, attention will be given only to Figure 3A, because of the possibility that holding the crabs in the laboratory may have altered their responses. In crabs tested on the day after collection (Fig. 3A) the white pigment was well dispersed on a white background, although it tended to concentrate somewhat at higher temperatures. On a black background the white pigment was dispersed at 15° C. but concentrated strongly when the temperature was raised. In the same experiments the black pigment on a black background was most concentrated at 15° C. and dispersed gradually with rising temperatures. The black pigment was more dispersed on a white background from 15° to 25° C. than it was on a black background, but it concentrated on the white background as the temperature was raised. The responses of the melanophores to black and white backgrounds intersected at about 25° C. It may be recalled that the melanophores did not respond clearly to background in the previous daytime experiments with light intensity (Fig. 1A). These experiments were run at 23° C., which was in the temperature range where the apparent effect of background was minimal.

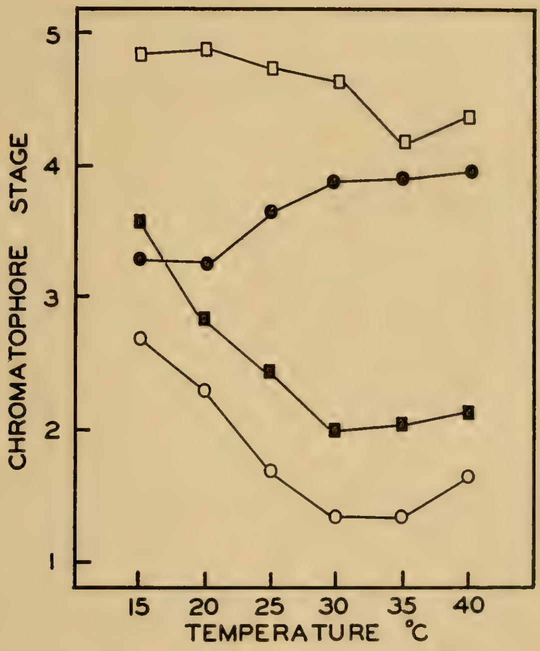

FIGURE 4. Relationships between pigment dispersion and temperature in *U. zacae*. Open circles, melanophores on white background; closed circles, melanophores on black background; open blocks, leucophores on white background; closed blocks, leucophores on black background.

Uca zacae

The results of two temperature experiments on the first and second days after collection were very similar and have been averaged for presentation in Figure 4. A secondary response of the melanophores and leucophores was evident at all tem-

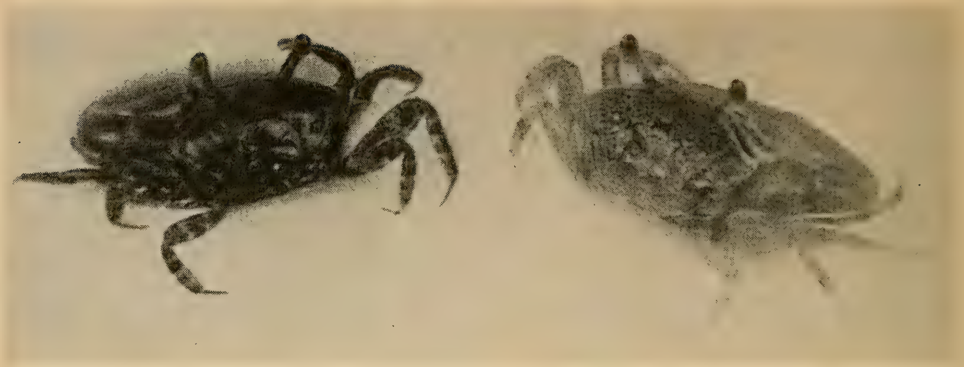

Figure 5. Male specimens of *U. zacae* at the conclusion of a temperature experiment. The crab on the left had been on the black background and the crab on the right, on the white background.

peratures from 15° to 40° C. The effect of the secondary response on the shade of the crab is shown in Figure 5, in which crabs from black and white backgrounds were photographed side by side in a white pan at the conclusion of an experimental series.

In the photograph, attention is called to another aspect of pigmentation in this species. Longitudinal white bands are evident across the merus, carpus, and propodus of the walking legs of the crab adapted to the black background. The banding results from the differential distribution of the melanophores on the segments of the walking legs. Conceivably, the pattern of white bands could serve to

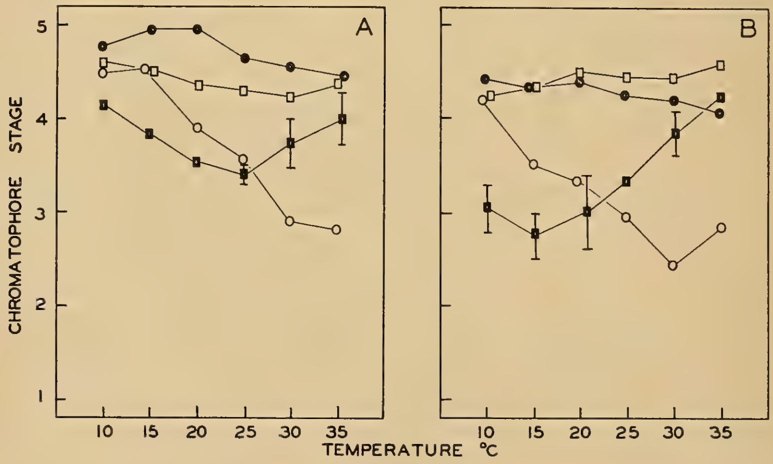

Figure 6. Relationships between pigment dispersion and temperature in specimens of *U. pugnax* in which the exposure to the series of temperatures began at (A) 10° C and (B) 35° C. Open circles, melanophores on white background; closed circles, melanophores on black background; open blocks, leucophores on white background; closed blocks, leucophores on black background.

camouflage the legs on a dark background by disrupting their surface. When the crab pales on a white background, the bands are no longer a conspicuous element of its coloration.

The melanophores of *U. zacae* showed a daily rhythm in their activity. Of 40 crabs examined at night at room temperature, 22° C., 38 had their melanophores in stage 1.

Uca pugnax

In Figure 6 are presented the average results of the experiments in which the same group of ten crabs was subjected sequentially to six temperatures. Whether presentation of temperatures began at 10° C. (Fig. 6A) or 35° C. (Fig. 6B), the black pigment was strongly dispersed at 10° C. but concentrated at elevated temperatures. The concentration was slight on a black background and greater on a white one.

The white pigment on a white background showed no consistent trend in response to temperature, except for remaining dispersed throughout the experiment. The greatest difference between the two types of experiments (exposure starting at 10° C. or 35° C.) was in the behavior of the leucophores on a black background. When the series began at 10° C. the white pigment concentrated as the temperature was increased to 25° and then dispersed somewhat at higher temperatures (Fig. 6A). With the series beginning at 35° the white pigment was most dispersed at 35° (Fig. 6B). The pigment then concentrated with decreasing temperatures, but the trend continued to 15° before reversing itself. These differences in the pattern of response of the leucophores were reproduced when the experiments were repeated, as shown in Figure 6. In the figure the closed blocks represent mean values for the leucophores on a black background, and the ends of the vertical bars show the values in each of the two experiments. In five of the 12 instances, the values were within 0.2 unit of one another.

Additional information on the response of the melanophores of *U. pugnax* to temperature has been provided by some experiments at night, when the black pigment is normally concentrated. Groups of ten crabs were exposed for one hour at 30 foot-candles to a single test temperature between 10° and 35° C. In a series of experiments it was found that the black pigment was more dispersed at lower temperatures than higher ones. These responses are similar to those obtained during the day phase of the color change cycle, but they also demonstrate that the black pigment actively disperses in response to low temperatures.

Comparison between species

From inspection of the graphs it is apparent that there were species differences in the direction of the trend in the chromatophoric responses to a series of temperatures. In *U. pugnax* the black pigment on both black and white backgrounds responded to increased temperatures by concentration, as did the black pigment of *U. pugilator* (Brown and Sandeen, 1948). By contrast, in both *U. g. herradurensis* and *U. zacae* the responses of the melanophores on the black and white backgrounds were more nearly mirror images of one another. Because of these opposing trends, the difference between dispersion of black pigment on black and white backgrounds was increased at higher temperatures.

In the two Costa Rican species the difference in dispersion of the white pigment on the two shades of background did not notably increase above 20° C. because white pigment on both backgrounds responded qualitatively in the same way to increased temperatures, that is, by concentration. In *U. pugnax* and, more conspicuously, *U. pugilator* (Brown and Sandeen, 1948) the difference between the dispersion of the white pigment on black and white backgrounds was reduced at higher temperatures. This was due largely to the tendency of the white pigment to disperse on a black background.

Discussion

This study has disclosed several differences in the pigmentary responses to light and temperature in species of fiddler crabs. These differences will be discussed here in an ecological context. This approach is prompted by the observation that the habitat of individual species of fiddler crabs tends to be very restricted (Crane, 1941). Type of terrain, degree of exposure to sunlight, and temperature are among the factors which appear to determine the distribution of a species. When such features of the habitat are constant for a species, there may well be correlated specializations in the chromatophoric system.

Uca pugilator

Crane (1943) has related *U. pugilator* to two subgeneric groups (her Groups 4 and 5) which include the most terrestrial and specialized of the fiddler crabs. Both behavioral and morphological adaptations for sandy terrain are known for this species. The adults and larvae show a strong preference for sandy over muddy substrata in the field and in choice situations in the laboratory (Teal, 1958). Structurally, the mouth parts are highly specialized for feeding on sand (Miller, 1961). Previous research (Brown and Sandeen, 1948) on *U. pugilator* may be interpreted in an ecological framework to provide an example of adaptation to a specialized habitat in the morphology and physiology of the chromatophores.

There appear to be morphological adaptations to sandy terrain in the chromatophores of *U. pugilator*. A comparison of *U. pugilator* and *U. pugnax* at Woods Hole, Mass., reveals that *U. pugilator* has fewer melanophores on the anterior face of the walking legs. Fingerman (1956) has noted the effects which are produced by the difference in the density of the melanophores. When black pigment is maximally dispersed, individual melanophores coalesce and produce a uniformly dark leg in *U. pugnax,* whereas in *U. pugilator* the melanophores remain as discrete entities against the paler portions of the exoskeleton and give the leg a speckled appearance. The adaptive advantage of the pattern of the melanophores in *U. pugilator* is probably that of concealment. Against the normal sandy background, a speckled walking leg would be less conspicuous than a homogeneously dark one. By contrast, the dark leg of *U. pugnax* is probably better adapted for concealment on the muddy substratum which this species prefers.

The physiological responses of *U. pugilator* appear to be consistent with morphological adaptations of the chromatophores for concealment on a sandy terrain.

The melanophores show only a weakly developed response to albedo (Brown and Sandeen, 1948). This weak response to albedo can be understood if the melanophores are serving to match the substratum through morphological patterning, rather than through physiological changes in over-all shading.[3] Although the melanophores may not show a clear response, the less opaque pigments of the erythrophores and leucophores surrounding the melanophores do respond strongly to the shade of the substratum. The red pigment disperses on a black background and concentrates on a white one, while the white pigment shows the opposite responses. The response of the erythrophores of *U. pugilator* to background dominates the daily rhythm to the extent that the rhythm can be expressed only in constant darkness (Brown, 1950). The relative significance of the albedo response and the daily rhythm of the erythrophores differs in *U. pugilator* from that in *U. rapax* and *U. maracoani* (Barnwell, 1963) and *U. g. herradurensis.* In these latter three species the daily rhythm clearly influenced any response of the erythrophores to background.

The weakness of the secondary response of the melanophores in *U. pugilator* is emphasized by the fact that it is overriden by the primary response (Brown and Sandeen, 1948). The net effect is that the black pigment is more dispersed on a white background than on a black one at the same incident light. This response is the opposite of what is generally observed in other adult animals. It was suggested that the primary response serves to screen the internal tissues from radiant energy (Brown and Sandeen, 1948).

Thermoregulation is another function which has been ascribed to the chromatophores of *U. pugilator* (Brown and Sandeen, 1948). As the temperature rises above 20° C. the black pigment concentrates and the white pigment disperses. In this way the absorptive area for radiant energy is reduced and the reflective area increased. This hypothesis is supported by measurements of the temperature of fiddler crabs insolated in air (Wilkens and Fingerman, 1965). The body temperature of "pale" crabs averaged 2° lower than that of "dark" crabs of the same species. Several writers (Parker, 1948; Waring, 1963) have emphasized that coloration can play a thermoregulatory role in air but probably not in water. The body temperature of a small aquatic poikilotherm would closely approximate the temperature of the surrounding water and would be little influenced by color change. Presumably, a thermoregulatory response in *U. pugilator* would be an adaptation to periods of terrestrial existence.

Uca galapagensis herradurensis, Uca zacae, and *Uca pugnax*

Crane (1941, 1943) has assigned the three species used in the present experiments, *U. g. herradurensis, U. zacae,* and *U. pugnax,* to a subgeneric group (Group 2) comprised of species which tend to be less brightly colored than *U. pugilator.* The dark shade of these species is probably correlated with their preference as a group for muddy over sandy habitats, since mud would be expected to have a lower albedo than sand.

In the three species of Group 2 studied here the black pigment was at some temperature more dispersed on a black background than on a white one at the same intensity of incident light. This was never true in *U. pugilator.* There were, however, large differences among the three species of Group 2 in the magnitude of the albedo response and the extent to which it was influenced by temperature. The very conspicuous albedo response of *U. zacae* is of particular interest. The behavior of this species does not conform with the statement by various authors that a physiological response to albedo normally has little influence on the coloration of *"Uca."* It should be recognized that this statement has been based on studies of only two species, *U. pugnax* and *U. pugilator,* out of perhaps 65 or more species comprising the genus *Uca.* Furthermore, while it is true that the melanophores of these two species do not exhibit pronounced responses to albedo, other pigments in the same crabs may show such responses. Admittedly, the melanophores are a major determinant of the coloration of the crab, but the more subtle effects produced by the albedo responses of the red and white pigments may have biological significance. This seems very possible in *U. pugilator,* as has already been pointed out.

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[3] This is not to exclude the possibility that the melanophores may respond to shade of background through morphological color change, as has been shown in *U. pugnax* (Green, 1964).

In the temperature experiments the pigmentary responses of *U. pugnax* resembled those of *U. pugilator* (Brown and Sandeen, 1948) in that they could be interpreted as thermoregulatory. The absorptive area for radiant energy was increased at lower temperatures and decreased at higher temperatures. However, the amplitude of the responses to temperature was somewhat lower in *U. pugnax* than in *U. pugilator.* For *U. pugnax* and other species of Group 2 which inhabit a dark substratum the thermoregulatory response of blanching at high temperatures would be antagonistic to the albedo response of darkening. In *U. pugilator,* on the other hand, thermoregulatory blanching would not render the crab so conspicuous against its normal sandy background, nor would the blanching be antagonized by a strong albedo response of the melanophores. It will be most interesting if the color changes in *U. pugnax* are of sufficient magnitude to play a thermoregulatory role. Both *U. pugnax* and *U. pugilator* have extended their ranges far into the temperate zone, to about 42° N lat., where they encounter greater daily and seasonal variations in environmental temperature than do the majority of species of fiddler crabs. Thermoregulation by physiological color change would appear to be a valuable capacity for species which must cope with short-term, but wide, variations in temperature.

The two tropical species, *U. g. herradurensis* and *U. zacae,* showed little evidence of what could be interpreted as a thermoregulatory response in their color change. The absence of a thermoregulatory response in these two species may be correlated with the narrower range of variation in temperature on the tropical coast compared with that on the temperate coast and with the higher thermal tolerance which tropical species have been reported to show over temperate species (Vernberg and Tashian, 1959). In the present experiments all tropical crabs survived the exposure for one hour to 40° C. whereas similar exposure can be lethal for *U. pugilator* (Brown and Sandeen, 1948; Orr, 1955; Teal, 1958). The effect of raising the temperature into the range normally encountered by these two tropical species was to enhance the albedo response. Apparently, the value of the albedo response in *U. g. herradurensis* and *U. zacae* outweighs any value which might be gained from the antagonistic thermoregulatory response.

There is, however, a circumstance in which the thermoregulatory role of blanching may be operative in tropical fiddler crabs. This occurs during the physiological color changes associated with display in the male. Crane (1941, 1944) has pointed out an evolutionary tendency in the fiddler crabs toward lightening of the carapace during display. The process has reached its culmination in the most terrestrial species of certain subgroups in which the carapace becomes dazzling white. This color change is one of several elements which increase the conspicuousness of the crab during display. But in addition to a possible role in advertising the crab, the extreme increase in reflective area should serve to reduce its body temperature. Such a function would be appropriate since the crab generally prefers to display in the direct rays of the sun.

From the standpoint of possible functions, the flexibility of the chromatophoric system in fiddler crabs is remarkable. As an example, physiological color changes may serve both to advertise a crab against the substratum during its display and a short time later to conceal it against the same background. Experiments with *U. pugilator* (Brown and Sandeen, 1948) showed that the stage of pigment dispersion is influenced simultaneously by a number of factors (intensity of light, albedo, rhythms, temperature), some of which are antagonistic and others, supplementary in their effects. In the present paper it has been demonstrated that the relative weights of these factors vary from species to species and that some of the variation can be related to the ecology of the crabs.

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SUMMARY

1. Chromatophoric responses to light and temperature were investigated in two tropical species of fiddler crabs, *Uca galapagensis herradurensis* and *Uca zacae*, and a temperate species, *Uca pugnax*.

2. The black, white, and red chromatophores of *U. g. herradurensis* showed a response to total illumination (primary response) in which the pigment dispersed with increasing light intensity.

3. In *U. g. herradurensis* there was a specific background, or albedo, response which resulted in concentration of the black and red pigments on a white background and their dispersion on a black background, while the white pigment showed the opposite response. The albedo response of the melanophores was present above 25° C. and increased as the temperature was raised to 40° C.

4. In *U. zacae* a strong albedo response resulted in darkening of the crab on a black background and blanching on a white background. The albedo response was most pronounced above 30° C.

5. In *U. pugnax* the black pigment concentrated with increasing temperature but the white pigment on a black background dispersed as the temperature was increased above about 20° C. In this regard the pattern of response to temperature in *U. pugnax* resembled that which had been published for another temperate species, *U. pugilator*.

6. Differences in the chromatophoric responses among species were emphasized. It was shown that some of these differences can be related to features of the habitats of individual species, such as type of terrain and temperature.

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OSMOREGULATORY CAPACITIES OF TWO CARIDEAN SHRIMPS, SYNCARIS PACIFICA (ATYIDAE) AND PALAEMON MACRODACTYLUS (PALAEMONIDAE)

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The osmoregulatory capacities of decapod crustaceans have been relatively well studied for the group as a whole. However, within the Section Caridea extensive studies on osmoregulation have been made on only one family, the Palaemonidae; this family is of interest because of the variation in habitat salinity shown by its various species. Of the 20 remaining caridean families only 2 have representatives presently inhabiting fresh water (FW): there are reports of 2 species of the genus *Alpheopsis* (Alpheidae) occurring in fresh waters (cited in Hedgpeth, 1957, p. 1157); almost all species of Atyidae appear restricted to fresh water. Holthuis (1963) reports the existence of 2 atyid species in brackish habitats.

As a result of zoogeographic studies the family Atyidae is considered an ancient, primarily tropical, fresh-water group. Epigean species inhabiting temperate regions are known from Japan, New Zealand, Europe, and the U. S. It is probable that the isolation of atyids from marine waters was established in the Jurassic (for reviews, see Ortmann, 1902, and Bouvier, 1925). According to this viewpoint atyids are fully as ancient residents of fresh water as the Astacidae, which have a fresh-water fossil record back to the Jurassic (Huxley, 1880). Two anatomic features suggest the atyid's primitive divergence from the common caridean stock: (1) the presence of exopodites on the pereiopods, a feature shared only by the oceanic Acanthephyridae; (2) the unique form of the chelipeds, both articles having a tuft of brushlike setae at their extremities.

In view of the abundance of atyids in certain localities, a study of osmoregulatory adaptations to provide comparison with the better known crayfishes seems particularly appropriate. While crayfishes have been the subject of studies on osmoregulation, atyids have not; practically the only knowledge of this family is systematic. The size and abundance of astacids in the U. S. and Europe make them more easily available than the relatively scarce atyids. In the U. S., only 2 of the 19 atyid genera recognized by Holthuis (1955) occur. The 3 extant species all have restricted distributions. The troglobitic genus *Palaemonias* is represented by 2 species, *P. ganteri* Hay, known only from Mammoth Cave, Kentucky, and *P. alabamae* Smalley, known from several caves in the southeastern U. S. *Syncaris pacifica* (Holmes), an object of the present study, inhabits certain permanent coastal streams in central California. *Atyäephyra desmaresti* Millet, originally inhabiting only circum-Mediterranean waters, now appears to be invading northern Europe (Vorstman, 1955), and is the only epigean atyid in Europe.

Of the 12 genera and about 180 species in the family Palaemonidae recognized by Holthuis (1950), the majority are reported to occur only in fresh water, although there are marine littoral and also completely euryhaline species described. Osmoregulation in 5 European palaemonids has been studied by Parry (1954, 1955, 1957). *Palaemon macrodactylus,* the second object of study in the present paper, has been introduced to the San Francisco estuarine system in the 1950s, probably from the Orient (Newman, 1963). It is clear from the physiological capacities and from the present distribution in the San Francisco area that this species is a euryhaline brackish-water form.

Hedgpeth (in press) provides a discussion of the distribution, salinity tolerance, and geological events in the range of *Syncaris pacifica.* Salient features of the biology of this species are presented here.

NATURAL HISTORY: SYNCARIS PACIFICA (HOLMES) 1895

The original generic name *Miersia* was replaced by *Syncaris* (Holmes, 1900). The present name should not be confused with the Division Syncarida which Packard (1886) proposed for certain Malacostraca showing a combination of sessile-eyed (edriophthalmous) and stalk-eyed (podophthalmous) characteristics.

Collections were made primarily in Salmon Creek, a small permanent coastal stream in Sonoma County, about 50 miles north of San Francisco Bay. In summer the bottom salinity of this stream is demonstrably brackish upstream for about two miles from its mouth. Adults have not been found in even the most slightly brackish parts of this or other streams. On 30 September 1965, several dozen adults were taken about $\frac{1}{4}$ mile above the point to which detectable salinity extends in the dry season. Collections during the winter rainy season 4 miles from the ocean showed that animals cling to vegetation with the pereiopods, out of the torrential current of the main stream.

A striking feature of *Syncaris* is its winter breeding pattern. Females collected late in September have become ovigerous within several days in the laboratory. From early October until mid-April practically all adult females are ovigerous. Duration of egg incubation is not known, but appears to be long in comparison with Nair's (1949) report of 17 days in *Caridina laevis* (Atyidae). Average egg dimensions are 1.3 × 0.9 mm., and only about 70 eggs are carried on the pleopods; in these respects *S. pacifica* is similar to other distinctly fresh-water crustaceans, having a reduced number of relatively large eggs compared to most marine decapods.

Although reliable population density estimates of an epibenthic organism capable of strong swimming movements are difficult to establish, in certain localities considerable numbers of individuals exist and *Syncaris* is a dominant organism in its ecosystem. Seining can yield 200 adults in a single haul in the most favorable habitats.

Feeding in *Syncaris* conforms generally to that described for 3 other atyids, *Caridina nilotica* and *C. africana* (Fryer, 1960), and *Atyoidia potimirim* (Müller, 1881). *Syncaris* is a detritus feeder and, along with the above species of *Caridina,* can be classified in Fryer's terminology as a microphagous chelate raptatory feeder.

EXPERIMENTAL METHODS

Animals were collected with a seine from their respective habitats and returned to the laboratory within 1 to 6 hr. and placed in aerated aquaria. Holding temperatures were 13° C. in winter, or room temperature (between 19° and 23° C.) between late spring and fall. Both species survive excellently in laboratory situations when kept in natural water and when provided with gravel, larger objects among which they can hide, and vegetation from the collection locality. *Palaemon* was fed bits of macerated fish, or live adult *Artemia*. No provisions for feeding *Syncaris* were necessary other than keeping vegetation and stream debris available. Consistent presence of material in the digestive tracts implied that the food available was adequate for experimental animals.

Body fluid samples were taken from as many sizes of individual as was convenient. Such an approach may have increased variation of results, but made maximum use of the material collected. Ovigerous female *Syncaris* were not used so that the drain on the rather small population was minimized. Although individuals survive sampling of fluid for both chloride and freezing point analysis, the data presented have come from individuals sampled only once.

Freezing points determined on a microcryoscope modified after that of Ramsay and Brown (1955) provided data for total osmotic pressure of blood and urine. Duplicate or triplicate samples were read to the nearest 0.01° C. on a calibrated Heidenhain thermometer, and the values averaged.

For collection of fluid for freezing point analysis the shrimp was wrapped in absorbent paper, placed ventral side up on a layer of transparent plastic over crushed ice, and viewed at 10–20 × with a dissecting microscope. The long appendages were held out of the way, the nephridiopores rinsed, dried with filter paper, and the sampling pipet then touched gently to the papilla bearing the antennal gland opening. Cannulation of the opening was avoided since the urine volume obtained by accumulation on the papilla is enough for analysis (approximately 0.01 mm.^{3}) and there is minimal chance of contamination of urine by blood. Urine of the two species showed different relationships to blood in different salinities, and this indicated that urine, as distinct from samples of either blood or medium, was being analyzed. After urine was collected the tip of a second antenna was cut off and blood collected in a similar manner.

Blood chloride concentration was determined with the Buchler-Cotlove chloridometer. The animal was dried thoroughly on the abdomen, wrapped in absorbent paper with the abdomen flexed and arthrodial membranes exposed for a quick puncture. Blood formed a drop on the dried hydrofuge integument; duplicate samples were taken in 1- or 2-microliter glass "Microcap" disposable capillary pipets (Kensington Scientific Corp.). Samples were rinsed directly into the acid reagent and titrated on the same day.

For convenience, environmental salinity is expressed in the Figures as per cent of local full strength sea water (540 meq. Cl^-/L., Δ2.08° C., 34‰). Note that lines of isotonicity with regard to chloride and freezing point do not coincide because chloride ion is only 90.2% of the total anionic component of sea water (data from Sverdrup, Johnson and Fleming, 1942, p. 173). A pure 0.588 *M* NaCl solution melts at −2.08° C., according to the International Critical Tables. The present estimate of sea water chloride concentration (540 meq./L.) is 91.9% of that found in a pure NaCl solution with Δ2.08° C. Comparison of the critically determined chloride concentration in local sea water having the observed freezing point depression (91.9%) indicates that the precision of the determinations is about ±1%.

EXPERIMENTAL RESULTS

The principal experimental results of this study appear in Figures 1–4, where blood chloride concentration and total osmotic pressure of blood and urine (Δ_i, by freezing point depression) are plotted against environmental salinity for *Syncaris pacifica* and *Palaemon macrodactylus.* Adjustment times in all cases have been at least 48 hrs. Stepwise increments of 15% SW were used to bring *Syncaris* up to high salinities, but more abrupt changes were used for *Palaemon.*

Figure 1 shows that in FW the blood chloride level in *Syncaris* is regulated at about 185 meq./L., rises in brackish water, becomes isotonic with the medium at about 290 meq./L., and in still higher salinities remains practically isotonic to the environment. Four days after the final chlorinity of 510 meq./L. (95% SW) was reached, all 8 animals showed a slight hypotonicity to the medium, equivalent to approximately 0.1% NaCl. This observation of hypotonicity shown by animals still alive after 4 days in salinities which would eventually be lethal is also seen in the freezing point data (Fig. 2).

Measurements on total osmotic pressure of blood and urine shown in Figure 2 support the conclusion that hyper-regulation is maintained by *Syncaris* below 50%

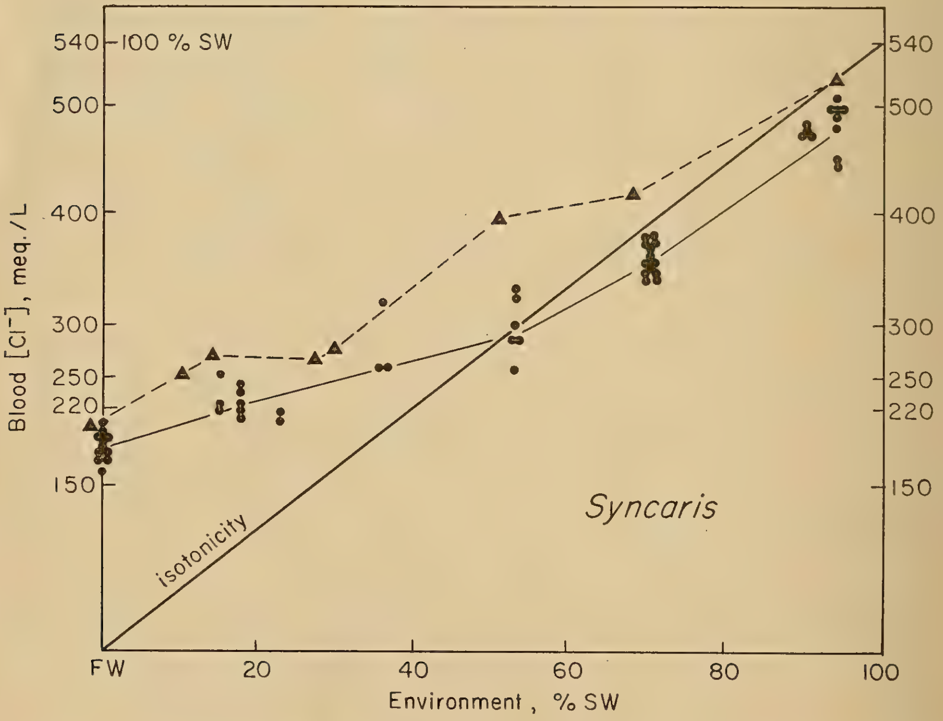

FIGURE 1. Blood chloride concentrations in *Syncaris pacifica* after at least two days acclimation to various strengths of sea water (environment, %SW). Each point represents duplicate determinations from an individual. ▲ represents means of adult freezing point data (Fig. 2).

SW, and further show that the antennal gland plays a role in osmoregulation by producing urine markedly hypotonic to blood. Between 10 and 30% SW (Δ_0 0.2–0.5° C.) blood concentration rises least steeply, from Δ_i 0.9° to 1.0° C. With increasing salinity, urine rapidly becomes isosmotic with blood, and concentrations of both fluids continue to increase. In the range 30–50% SW there appears to be a maximum rate of change of urine concentration. In 50% SW urine and blood are isosmotic at about 125% of medium concentration. In 70% SW urine, blood, and environment are in equilibrium. There is slight hypotonicity with respect to total concentration in 95% SW as has already been seen for chloride ion.

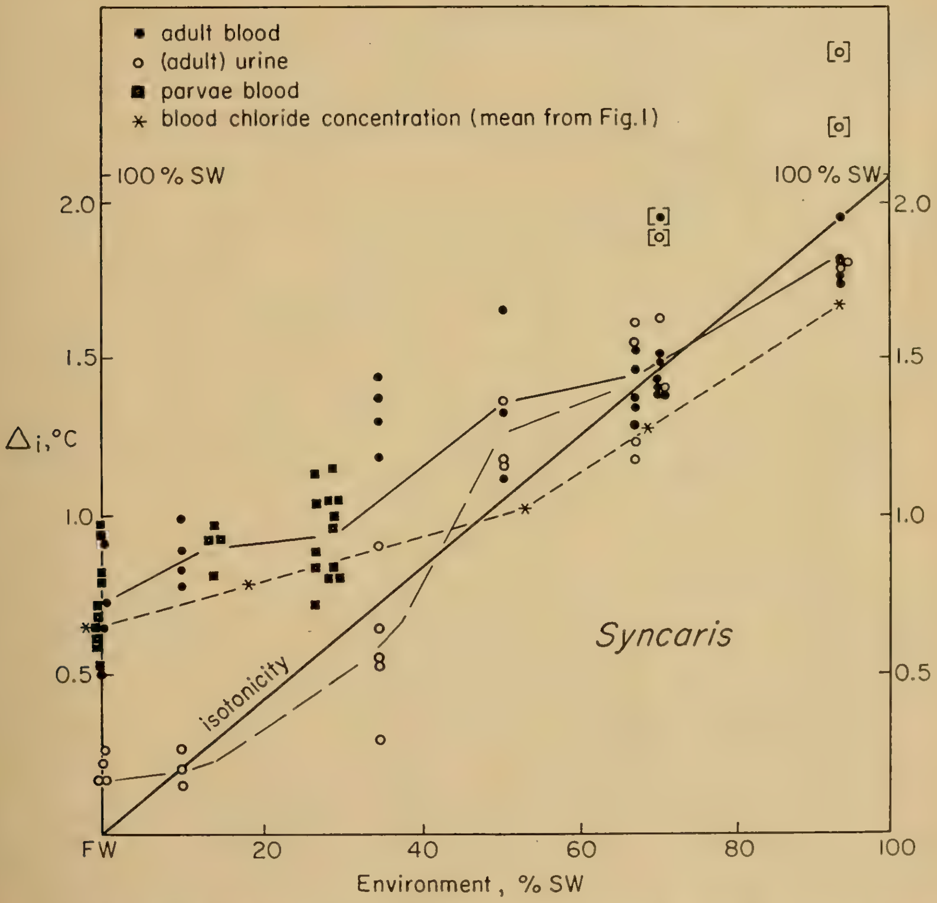

FIGURE 2. Blood and urine concentration (Δ_i) in the fresh-water shrimp *Syncaris pacifica* after at least two days acclimation to various strengths of sea water (environment, %SW). Solid lines connect means of the Δ determinations. Dotted lines connect means of blood chloride levels (data from Fig. 1), expressed in Δ equivalents. Each point represents duplicate determinations from an individual. Dots (●) represent adults, squares (■) represent "parvae" stages, circles (○) represent urine, and asterisks (*) represent means of adult blood chloride concentrations. Data in brackets have not been included in determination of means.

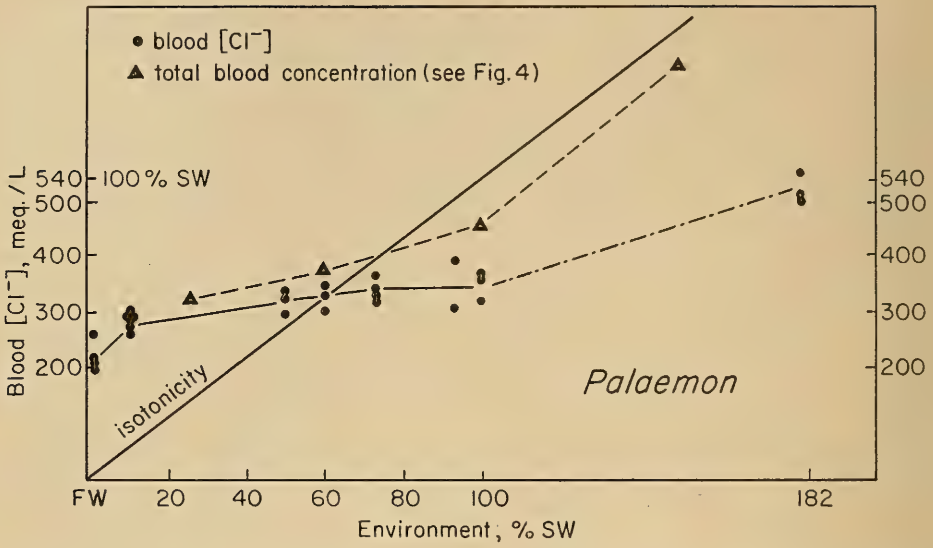

FIGURE 3. Blood chloride regulation curve in the euryhaline shrimp *Palaemon macrodactylus.*

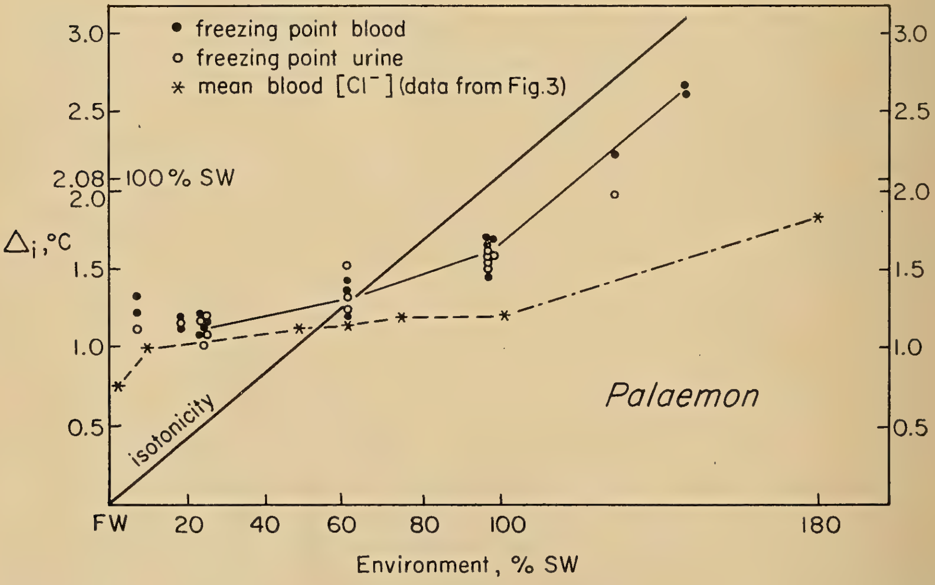

FIGURE 4. Blood and urine concentration (Δ_i) in the euryhaline shrimp *Palaemon macrodactylus* in various salinities (environment, %SW).

Blood samples from "parvae" (young postlarval size class individuals; average length about 10 mm.) are shown as separate points in Figure 2. In low salinities the level of regulation appears to be very similar in parvae and adults.

Survival of *Syncaris* adults was poor when 6 individuals were placed directly back into FW following sampling after 3 days of adjustment to 95% SW. Previously-sampled individuals acclimated to 70% SW survived such abrupt transfers back to FW. Although a large-scale test has not been made, it is probable that gradual reacclimation of this species from the highest tolerable salinities back to FW would be successful.

Figure 3 shows the blood chloride regulation of *Palaemon macrodactylus*. Animals collected in November in water of only 11 meq. Cl^-/L. (2% SW) hyper-regulated blood chloride at a level of 210 meq./L., only slightly greater than that shown by *Syncaris* in FW (185 meq./L.). Between 10 and 100% SW, chloride regulation in *P. macrodactylus* is very strong, increasing only from 285 to 355 meq./L. Blood chloride is isotonic with an environment of 318 meq. Cl^-/L., or 59% SW.

Unlike the dilute urine of *Syncaris*, urine of *Palaemon macrodactylus* appears to be isosmotic with blood in all salinities tested, as shown in Figure 4. Between 20 and 100% SW, strong regulation of total blood concentration is shown by the slight slope of the curve, increasing only from Δ_i 1.19° to 1.65° C. as environment increases from Δ_o 0.5° to 2.02° C. (24–97% SW). Body fluids are isosmotic with the medium in 72% SW. However, total blood concentration, although it continues to be hypo-osmotic to medium, does not appear to be under such precise regulation in hyper-saline media as is shown for chloride ion (Fig. 3). Since the respective adjustment times were 3 weeks for animals shown in Figure 3, and 3 days in Figure 4, further tests are needed to determine the meaning of the marked difference in chloride and Δ_i regulation in hypersaline conditions.

DISCUSSION

It has been mentioned that the Atyidae, like crayfishes (Astacidae), appear restricted to fresh water. The data for *Syncaris* suggest that, in terms of blood and urine relationships, atyids and crayfishes are more similar to each other than they are to other decapods which have been studied. In fresh water, *Syncaris* urine is *ca.* 25% of blood concentration while in crayfishes the urine concentration is *ca.* 10% that of blood. Both of these types are in an advanced state of adaptation and profit osmotically by excretion of markedly hypo-osmotic urine.

It has been suggested by Ortmann (1902) and Hedgpeth (1957) that the subfamily Palaemoninae is in a transitional status from marine ancestry to fresh-water invaders. In one of the few studies of osmoregulation in a palaemonid which spends its entire life cycle in fresh water, Parry (1957) found that *Palaemonetes antennarius* has a blood concentration in the range shown by crayfishes and *Syncaris*, but has only a slightly hypo-osmotic urine (Δ_B 0.75° ; Δ_U 0.67° C.). Dobkin and Manning (1964) found that the fresh-water *Palaemonetes paludosus* has a relatively low blood concentration (Δ_B 0.87° C.) in the natural fresh-water environment, lower than its euryhaline congener, *P. intermedius* (Δ_B 1.10° C.) when the latter is in 10% SW, the lowest salinity tested. Unfortunately, urine was not investigated in the latter study. Comparative information on osmoregulatory adaptations in species of *Macrobrachium* would be most interesting. In this large palaemonid genus there are species which apparently hatch larvae in brackish water (*M. ohine,* Gunter, 1937; *M. carcinus,* Lewis and Ward, 1965) as well as species in which the entire life cycle is spent in fresh water. Correlations between osmoregulatory adpatations and habitat history of a species might be found.

A generalization which emerges from studies of regulatory responses to high salinities shown by decapods which normally spend their entire lives in fresh water is that almost all types eventually become isosmotic with salinities appreciably higher than the level at which blood concentration is regulated in fresh water; however, times required for adjustment vary. From the few studies of fresh-water carideans, it appears that such shrimp become isosmotic within 48 hours after transfer into brackish media. *Palaemonetes paludosus* is isosmotic in high salinities after a 42-hour adjustment period (Dobkin and Manning, 1964). Apparently this palaemonid has lost the capacity to hypo-regulate in high salinities. *Syncaris* blood becomes practically isosmotic with medium within 48 hours when a series of 15% SW increments is used to bring experimental animals to high salinities.

On the other hand, Duval (1925) showed that when the fresh-water crab *Potamon edulis* was exposed to high salinities (Δ_0 1.8° C.) at least 20 days elapsed before the blood was isosmotic with the medium. Since all fresh-water crabs studied have a high blood concentration, with urine isosmotic to blood, it is not surprising that mechanisms other than elaboration of dilute urine operate to effect hyper-regulation in the natural environment. Strongly reduced sodium permeability has been found in several fresh-water crabs, at least an order of magnitude lower than that reported for brackish-water crabs (Shaw, 1961).

Members of the Nephropsida (= Astacura), the third group of decapods with fresh-water representatives, have been most thoroughly studied, and four types of regulatory adjustment to the osmotic problems of fresh water have been demonstrated: considerable lowering of blood concentration, excretion of hypotonic urine, active ion uptake by gills (see *e.g.,* Bielawski, 1964), and strongly reduced ion permeability. Direct evidence for the latter comes from Shaw's (1959) report of a sodium loss rate in *Astacus pallipes* an order of magnitude lower than the same author found in the FW crab *Potamon niloticus,* and Gross's (1957) report showing that the isolated carapace of *Procambarus clarki* is less permeable to salts than any of the brackish-water or marine species tested. Indirect evidence for reduced permeability comes from time-course studies on changes in blood concentrations in various crayfishes upon exposure to brackish water. Kerley and Pritchard (1967) show that after 2 days the blood of *Pacifastacus leniusculus* is distinctly hypotonic to 70% sea water, and the blood does not become isosmotic with the environment until 20 days have elapsed. Similarly, Herrmann (1931) found that blood of *Astacus astacus* is hypotonic after a 4-day exposure to high salinity (Δ_i 1.54° C., Δ_0 1.8° C.); death occurred shortly thereafter, but the progressive rise in blood concentration seen during the first 4 days suggested that, had the animals survived, osmotic equilibrium with the environment would have been reached. L. C. Thompson of this laboratory has found that *Procambarus clarki* survives well in 70% SW and that blood concentration gradually rises, until after 20 days the blood is isosmotic with the medium.

It is concluded that, upon exposure to increasing salinity, normal osmoregulatory mechanisms of fresh-water decapods continue to maintain the blood hyperosmotic to low salinities. But in higher salinities the animals either hypo-regulate by eliminating excess ions and conserving water, or conform to the environment. Since the hypo-regulatory response required is the opposite of adaptations elicited by naturally operating selective factors, it is understandable that fresh-water decapods are unable to effect sustained hypo-regulation in high salinities. The concept of a "salinity tolerance range" is an experimentally observed capacity, since the forms under consideration complete their entire life cycle in fresh water.

This paper is based on a thesis submitted to the Department of Zoology, University of California, Berkeley, in partial fulfillment of requirements for the M.A. I thank Dr. Ralph I. Smith for general guidance. Dr. A. C. Broad, Western Washington State College, Bellingham, also gave advice and encouragement. Financial support was provided by an N. S. F. Cooperative Graduate Fellowship. A portion of the study was conducted at the Bodega Marine Laboratory, and I thank its Director, Dr. Cadet Hand, for providing space and equipment. Dr. Joel Hedgpeth kindly allowed me to read and cite his paper on *Syncaris*. Numerous fellow students have given assistance; in particular I thank Mr. Armand Kuris for his generous field help, and Mr. L. C. Thompson for discussion of the literature on crayfish osmoregulation.

SUMMARY

1. *Syncaris pacifica* (Atyidae) is restricted to fresh water, like most members of the family. The striking osmotic adaptation in this shrimp is elaboration of urine markedly hypo-osmotic to blood. In fresh water, the following average body fluid concentrations were found: blood, Δ_i 0.73° C. (F. P. depression), chloride, 185 meq./L.; urine, Δ_i 0.18° C. This is the first report of dilute urine in any decapod other than crayfishes.

2. *Palaemon macrodactylus* (Palaemonidae) is a strong regulator over the range 2–150% SW, and inhabits a wide range of salinities in the San Francisco Bay estuarine system. Blood and urine are isosmotic throughout the tested range of salinities. Hyper-regulation occurs below 60% SW, and hypo-regulation in higher salinities. Between 20% and 100% SW, Δ_i increases only from 1.19° to 1.65° C., as environmental salt concentration increases from Δ_0 0.50° to 2.08° C.

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TUBULARIA REGENERATION: RADIAL ORGANIZATION OF TENTACLES, GONOPHORES, AND ENDODERM

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The marine hydroid *Tubularia* is frequently used as an experimental animal in studying the axial organization of hydroids (Berrill, 1961; Tardent, 1963). Yet virtually nothing is known about the radial organization of this or other hydroids, despite the fact that the phylum Coelenterata is characterized by the radial construction of its representatives.

From its outward aspects, the tubular body of *Tubularia* shows a radial organization in the even spacing of tentacles in two whorls, and in the somewhat less regular arrangement of gonophores in an intermediate ring. Inside the hydranth, regularly spaced longitudinal ridges ("taeniolae," Hamann, 1882) in the endoderm give the cross-section of the hypostome lumen a stellate appearance. Finally, the stalk has a cartwheel pattern in cross-section due to its partition into several longitudinal channels by bridges of endodermal cells.

In this paper we describe how these several visible manifestations of radial organization are developmentally related to each other during regeneration of a hydranth from a segment of stalk.

Methods and Materials

Tubularia crocea (Agassiz) was collected on floats in Newport Bay, California. *Tubularia larynx* (Allman) was collected from a buoy at Arcachon, France. Regeneration was carried out at 16° C. Animals were fixed for 12 hours in the following solution: 5 ml. formalin; 2 ml. glacial acetic acid; 0.5 g. $K_2Cr_4O_7$; 25 ml. alcohol; 18 ml. distilled water; 50 ml. sea water. Before fixation, tissue covering the two ends of regenerating stalks was punctured. Serial paraffin sections 10 μ thick were cut perpendicular to the longitudinal polyp axis, and stained with Ehrlich's hematoxylin and Biebrich Scarlet.

About 50 stalks and several hundred regenerating polyps were analyzed, and all the observations presented apply equally well to both species of *Tubularia*. All illustrations were prepared from specimens of *T. crocea*.

Results

A. Radial organization of the normal stalk

The stalk of *Tubularia* is organized radially before regeneration begins. The cavity of the stalk is partitioned into two or three longitudinal channels by endo-

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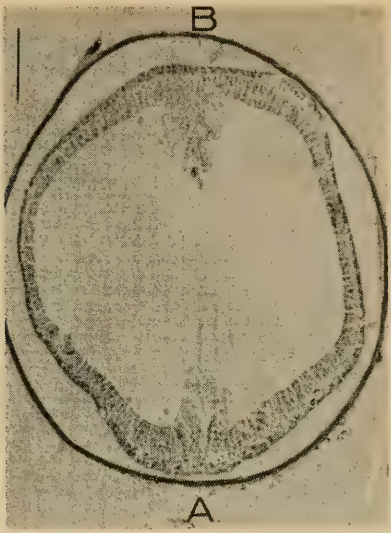
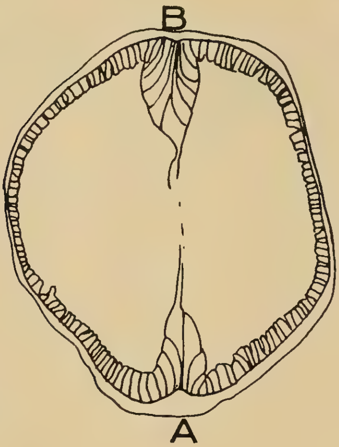

B
A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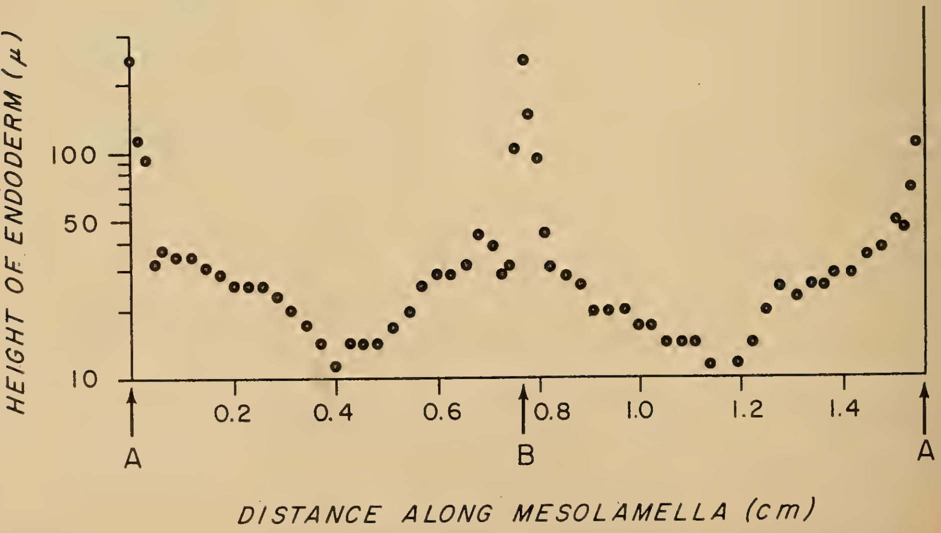

HEIGHT OF ENDODERM (μ)
100
50
10
0.2
0.4
0.6
0.8
1.0
1.2
1.4
A
B
A
DISTANCE ALONG MESOLAMELLA (cm)

dermal septa, cellular bridges stretching across the lumen. However, the participating cells should not be considered as developmentally unique in a column of otherwise homogeneous cells. In Figure 1 it can be seen that there is a gradation of endodermal cell structure and size from the septal radius, where endodermal cells are tall and vacuolate, to the low interseptal regions, where cells are short and densely staining. This suggests that the endodermal septum, while itself narrow, is a manifestation of a broader radial organization or gradient within the stalk endoderm. Different stalks vary greatly in size and abundance of food inclusions, but consistently show these radial gradations. The width of the septum, sharpness of the gradation and lowest height of the endoderm are extremely variable, however.

This radial order is also visible in the ectoderm, where the density of interstitial cells and nematocytes is slightly higher in the positions adjacent to the endodermal septa.

The term "septal plane" will be used in this paper to refer to the radial directions in which the septa meet the wall; in *T. larynx* and *T. crocea* there were 2 to 4 septal planes in most mature polyp stalks.

B. *Initiation of tentacles*

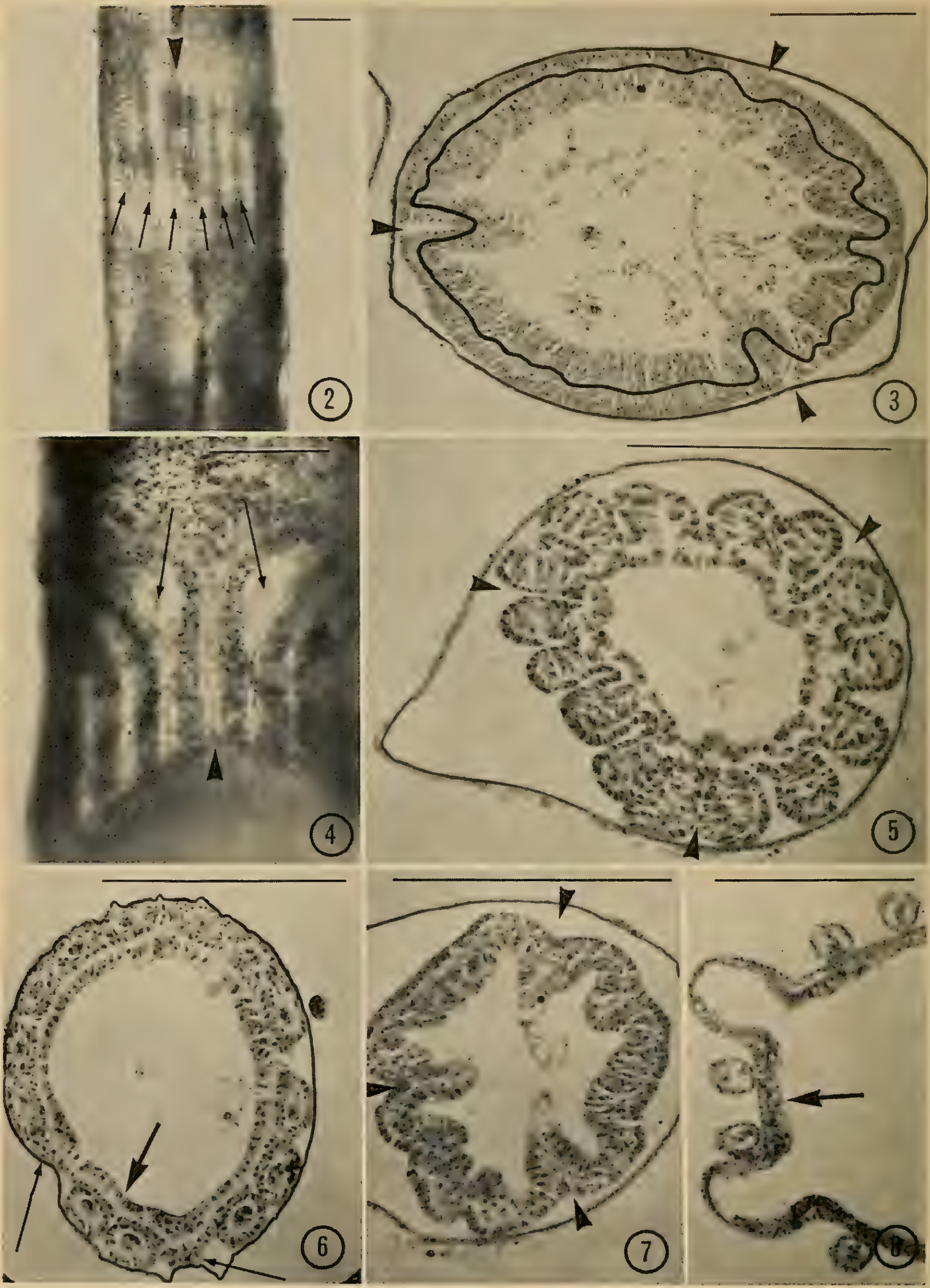
During regeneration, tentacles originate as two whorls of longitudinal ridges. The septal planes are always intertentacular (Figs. 2, 5), in both proximal and distal tentacle whorls. This relationship becomes apparent quite early in regeneration. The mesogloea first becomes indented along the septal planes (Fig. 3). These grooves indicate the primary intertentacular positions. On both sides of each groove, the mesolamella bulges outward slightly, defining the positions of the adjacent tentacle ridges. Subsequently the mesogloea, as seen in cross-section, becomes wavey all around the stalk (Fig. 3). Each flute in the mesolamella indicates the position of a developing tentacle ridge.

As regeneration proceeds, the septa gradually disappear from hydranth regions. However, they persist for some time as fine tenuous strands (see Fig. 5) often lost during histological preparation. Even in newly everted regenerated hydranths, the positions of the original septa are indicated by slightly more dense pigmentation.

C. *Gonophore positions*

Gonophores form during the regeneration of mature stalks. They usually appear between proximal tentacles, near the distal tips, shortly after the ridges begin to separate from the stalk as independent tentacles. Gonophores arise in pairs, one on each side of every septal plane (Figs. 4, 8). The number of gonophores on a young regenerate is thus twice the number of septal planes. Gonophores almost invariably arise between the first and second tentacles (Figs. 4, 8), counting in each direction from the septal plane; they very occasionally appear as far away from this plane as directly over the second tentacle, or as close as the first tentacle.

FIGURE 1. Radial organization in *Tubularia* stalk. 1a. Photograph of section analyzed. 1b. Tracing of photograph in Figure 1a, showing pattern of endodermal cells and the outline of ectoderm (the perisarc is not shown). 1c. Height of endodermal epithelium measured at intervals around the circumference. The septal planes indicated by the abscissa points A and B are indicated by letters on the photograph in Figure 1a. The positive direction along the abcissa corresponds to a counterclockwise direction in the photograph. Scale represent 100 μ.



FIGURES 2–8. Scales represent 200 μ.

After a regenerated hydranth has everted from the perisarc, additional gonophores are produced. Their positions are less regular, although generally the first ones form roughly midway between existing pairs of gonophores.

The earliest appearance of gonophores cannot be correlated with any histological character other than the septal and tentacular positions. They arise from cells which are histologically indistinguishable from those in the surrounding hydranth wall. The first histological sign of gonophores is that the ectodermal cells in regions of developing gonophores appear to adhere to the perisarc (Fig. 6), and endodermal cells shortly become noticeably more basophilic.

D. Formation of the endodermal ridges

The endodermal ridges, or taeniolae, of the hypostome arise in intertentacular positions, both above and below the distal row of tentacles. There is continuity in the histological structure from taeniolae to tentacular regions.

The most prominent taeniolae in early regeneration are along the septal planes (Fig. 7); these apparently arise directly from the tall septal cells. Other taeniolae appear later. The formation of these ridges also presumably involves either cell proliferation or cell movement, as taeniolate cells are more densely packed (per unit area of mesolamella) than are inter-taeniolate cells. In some specimens, containing mitotic figures, most dividing endodermal epithelial cells are in inter-taeniolate positions. Taeniolae are also rich in gland cells whereas stalk septa lack these.

DISCUSSION

In this paper it is reported that the regenerating *Tubularia* hydranth shows definite patterns of radial organization which are related to the pre-existing structure of the stalk. Some other reports have also indicated that radial, in addition to axial organization is patterned in a variety of hydroids. Cohen (1952) observed that the grafting of *Tubularia* stalk segments is generally successful only if the

FIGURE 2. Side view of tentacle-ridge stage of regeneration. Proximal tentacle ridges are indicated by small arrows. Large arrow indicates a septal plane, visible as a dark longitudinal line running between tentacle ridges.

FIGURE 3. Cross-section of pre-ridge stage. The 3 septal planes are indicated by arrows. The mesolamella is traced out in ink on this photograph.

FIGURE 4. Side view of a regenerate in which proximal tentacle ridges are about half separated from the wall. A pair of gonophores, indicated by small arrows, lies between the first and second tentacles on each side of a septal plane (large arrow).

FIGURE 5. Cross-section through whorl of distal tentacle ridges. The three septal planes (arrows) are intertentacular. Remnants of the septa are visible.

FIGURE 6. Cross-section through a regenerate at an age when gonophore positions are first indicated by the ectoderm adhering to the perisarc (small arrows). The adjacent endoderm is slightly more basophilic than other cells. These gonophore rudiments are symmetrically located between the first and second tentacles on each side of the septal plane (indicated by large arrow). (Gonophores on opposite side of stalk are off the plane of section.)

FIGURE 7. Same specimen as shown in Figure 5, but below the distal tentacles. Large taeniolae are present along the three septal planes (arrows); similar ones are beginning to form along other intertentacular planes.

FIGURE 8. Cross-section of everted regenerated hydranth. Gonophores, apparent as evaginations of the body wall, are between first and second tentacles on each side of the original septal plane (arrow).

endodermal septa of the two pieces are aligned. In *Corymorpha*, whose hydrocoel is partitioned into many longitudinal channels, the endodermal channels of two grafted stalk portions apparently become aligned quite early in regeneration (see Okada, 1927). The radial localization of mitotic cells in *Tubularia* and *Hydractinia* (Campbell, 1967) and the histological structure of most hydroids (Hamann, 1882; Campbell, 1967) also indicate the presence of a major radial morphogenetic order in the polyp. Some colonial hydrozoans, such as the one-tentacled *Monobrachium* (Hand, 1957) and the two-tentacled *Proboscidactyla* (Hand, 1954) have an asymmetrical polyp whose radial organization bears a constant orientation to the colony pattern.

There appear to be basic developmental differences between the radial and axial organizations of polyps. The axial differentiation of polyps is most easily considered in terms of a linear developmental hierarchy. For example, the order of axial regions in the polyp (hypostome, distal tentacles, reproductive region, and proximal tentacles) shows virtually no rearrangements in the hydrozoans, despite modifications of evolution, polymorphic development and experimental manipulation. Furthermore, regeneration studies indicate that the developmental stability of any axial region is generally dependent on the next most distal region. The axial hierarchy of polyp regions thus appears to be a stable and fundamental one.

Radial organization, on the other hand, appears as whorls of equivalent developmental domains within a certain axial level of the tissue. In the region of the hypostome, the domain structure is manifested in the placement of tentacles and in the taeniolate organization of the endoderm (Campbell, 1967). The presence of domains in the stalks of *Tubularia* and *Corymorpha* is indicated by the presence of endodermal septa. Each axial polyp level has its characteristic radial organization, although variations do occur in different groups of Hydrozoa.

Radial domains seem to be mutually inhibitory. Tentacles commonly arise midway between existing ones, and the domains characteristically maintain a regular spacing around the column. The appearance of new radial modes typically follows radial growth of the column (in *Hydra*, tentacle number is roughly proportional to size [Parke, 1900]; stalk septa in *Corymorpha* are related to girth [Torrey, 1907]), indicating that domains have a constant stable size, which seems generally to increase slightly along with the animal's diameter. Also, radial sectors cut from a polyp are often regenerated only very slowly, perhaps not faster than the normal growth rate, while missing axial regions are rapidly replaced by regeneration processes. Apart from basic characteristics such as these, we know little about the developmental nature of radial domains.

Hydroids are being extensively used to study only axial developmental processes, yet the phylum Coelenterata is classically characterized by the radial organization of its representatives. It is thus appropriate in studying hydroid development that one consider the radial as well as the axial patterns of morphogenesis. The final radial or axial order of polyps may result from the interaction of the two developmental systems.

SUMMARY

1. The stalk of *Tubularia* is divided by several cellular septa. There is a gradient in cell height from septal to interseptal planes. Septal cells can thus be

interpreted as manifestations of a single (repeating) developmental or histological gradient, rather than as unique cells in an otherwise homogeneous epithelium.

2. When a hydranth regenerates from a segment of *Tubularia* stalk, the tentacles, gonophores and hypostomal ridges (taeniolae) arise in relation to this preexisting radial organization of the stalk.

3. Tentacles appear in such positions that endodermal septal planes are intertentacular. Gonophores form in pairs, one on each side of every septal plane, between the first and second tentacle. Taeniolae arise along the septal, and later along other intertentacular planes.

4. These and other relations suggest that the visible radial order of the hydroid polyp is a manifestation of a fundamental, domain-like radial tissue organization.

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A SURVEY OF THE EFFECT OF OSMOTIC DILUTION ON FREE AMINO ACIDS OF VARIOUS POLYCHAETES [1]

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Low molecular weight organic nitrogen compounds contribute a large proportion of the total intracellular osmotic activity in many invertebrates (see Florkin *et al.*, 1964, for literature review). The polychaetes which have been studied in this respect are *Arenicola marina* (Duchâteau-Bosson *et al.*, 1961), *Nereis diversicolor* and *Perinereis cultrifera* (Jeauniaux *et al.*, 1961), all of which show a decrease in tissue free amino-acid levels during periods of osmotic stress in reduced salinities. So far little attempt has been made to relate the decrease in tissue free amino acids to possible changes of free amino acids in the coelomic fluid during the period of osmotic adjustment in any marine invertebrate. It was therefore considered worthwhile, during an investigation of the free amino-acid concentrations in the coelomic fluid and body wall of various polychaetes adapted to full-strength sea water (Clark, 1968), to observe the effects of osmotic dilution on the amino-acid levels of both fluid compartments.

To elucidate completely the manner in which a particular animal adjusts the composition of its body fluids to meet varying salinities requires measurements of both the equilibrium achieved at each dilution and the rate at which this equilibrium is reached. Neither the time nor number of animals available for this investigation permitted an analysis of these parameters, and, indeed, for several of the species examined it was not known whether any adjustment would take place at all. The present study is therefore a survey of the types of adjustments made by various polychaetes during specified periods of osmotic stress. Animals were exposed for 1, 2 or 4 days to dilutions of 75% or 50% sea water and changes in the free amino acids in the body wall were measured in relation to changes in tissue hydration. Most species show an increase in tissue water, which accounts for most of the fall in free amino acids in the body wall during osmotic stress, although in some cases energy-requiring processes appear to contribute to the decrease as well. Simultaneous measurements of changes in amino acids in the coelomic fluid suggest that not all species transfer amino acids between fluid compartments in the same way.

MATERIAL AND METHODS

Ten species of animals from Puget Sound were studied: *Nephtys caecoides, N. ciliata, Nereis vexillosa, Eunice biannulata, Abarenicola pacifica, Amphitrite*

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cirrata, Neoamphitrite robusta, Thelepus crispus, Aphrodite japonica and *Travisia pupa*. The specific localities from which the animals were collected are detailed elsewhere (Clark, 1968). The animals were maintained in running sea water tables at 8 to 10° C. For osmotic stress treatments, small groups of each species were placed directly into large fingerbowls lying in the water table, and containing 1 liter of either 100% (30 to 32 parts per thousand salinity), 75% or 50% local sea water diluted with glass-distilled water, which was changed every twelve hours. All species were tested at both dilutions since in many cases the ability to survive osmotic stress was not known. Treatments lasted from 1 to 4 days, and although some species showed obvious negative reactions to being placed in 50% sea water (see results) almost all individuals of each species survived the experimental period. The number of animals available did not allow a study of the ability to recover from treatments in marginal cases.

Measurements of total ninhydrin-positive nitrogen and of ammonia nitrogen of both body wall and coelomic fluid samples were carried out by the methods already described (Clark, 1964; 1968). Amino-acid nitrogen (AAN) values were obtained by difference.

RESULTS

Not all of the worms react equally well to dilute sea water. Usually there is no behavioral response to 75% sea water either immediately or after 24 hours, although *Eunice* moves about vigorously at first, becoming quiet again in a few minutes. *Abarenicola* appears to increase slightly in body volume. Only the terebellids show signs of negative reactions: *Neoamphitrite* appears the same in 75% sea water as in 100%, but although *Thelepus* spreads its tentacles again within a few minutes after being transferred to 75% sea water, by 24 hours these are partially contracted. *Amphitrite* shows the greatest reaction, with slight swelling of the body, and tentacles never more than half extended.

In 50% sea water all three species of terebellids contract their tentacles over the branchiae. In *Amphitrite* the branchiae become blanched and non-motile, the entire body swells and the rate of pumping becomes very slow. Response to touch is absent. Other species, including *Abarenicola, Eunice* and *Nereis*, show some increase in body volume. *Aphrodite* becomes extremely distended ventrally a few minutes after being placed in 50% sea water, but this swelling is reduced to a negligible amount after about an hour. Both species of *Nephtys* thrash about, with repeated proboscis eversion, when put into 50% sea water, but later become flaccid. *Eunice*, after an initial period of hyperactivity, becomes quiescent and shows reduced responsiveness to stimuli. *Nereis* is able to build tubes and move equally well in 50% as in 100% sea water. *Travisia* shows no external signs of osmotic stress and no swelling, probably because of resistance to deformation from the tough musculature and thick cuticle of the body wall.

The results of the effects of osmotic stress on free amino-acid levels in the coelomic fluid and body wall are shown in Table I. The values are derived from the difference between the total ninhydrin-positive nitrogen and ammonia nitrogen estimates on each sample. There were no consistent variations in ammonia levels in experimental animals compared to animals in 100% sea water (Clark, 1968). In some samples, ammonia values increased slightly, in others they appeared to

TABLE I

Amino-acid nitrogen levels in coelomic fluid and body wall of polychaetes in different concentrations of sea water. "Active" change (ΔAAN) in tissue amino-acid levels is derived from the difference between the observed value and the value expected due to increased hydration. The calculated rise in freezing point (Δ°C.) due to decrease in amino-acid levels is based on the observed values

Species	Days of expt.	No. worms	% sea water	Coelomic fluid	Body wall				
				AAN mg./100 ml.	% water	Obs. AAN mg./100 g. H_2O	Exp. AAN mg./100 g. H_2O	"Active" ΔAAN mg./100 g. H_2O	Calc. Δ°C. from total ΔAAN
Nephtys caecoides	2	10	100	44.55	73.47	236.7			
	2	8	75	38.25	76.52	233.8	201.1	+32.7	<+0.01
	2	9	50	52.77	78.08	140.8	184.0	−43.2	+0.13
Nephtys ciliata	1	5	100	71.34	73.35	291.8			
	1	6	75	62.40	74.40	181.3	276.3	−95.0	+0.15
	1	3	50	69.90	71.42	259.2	321.3	−62.1	+0.04
	2	4	100	35.43	72.12	341.3			
	2	6	75	19.14	75.66	239.4	284.0	−44.6	+0.14
	2	3	50	68.91	73.52	223.4	317.9	−94.5	+0.16
Nereis vexillosa	1	8	100	19.68	74.25	463.4			
	1	8	75	29.01	77.77	374.1	382.0	−7.9	+0.12
	1	7	50	13.41	82.70	241.1	279.6	−38.5	+0.29
	2	10	100	18.13	75.28	486.0			
	2	6	75	13.41	79.84	378.5	373.7	+4.8	+0.14
	2	7	50	18.74	83.72	223.6	287.8	−64.2	+0.35
	2	9	100	—	74.13	491.2			
	2	8	75	17.55	77.36	306.6	411.9	−105.3	+0.24
	2	8	50	43.42	81.85	222.6	312.1	−89.5	+0.36
Eunice biannulata	1	9	100	66.72	76.07	330.7			
	1	9	75	90.39	79.11	369.0	277.6	+91.4	−0.05
	1	9	50	63.14	80.78	238.5	250.1	−11.6	+0.12
	2	8	100	72.60	74.67	358.9			
	2	9	75	48.85	77.27	199.8	311.2	−111.4	+0.21
	2	7	50	65.84	83.80	201.9	204.5	−2.6	+0.21
Abarenicola pacifica	1	4	100	5.50	80.14*	385.5			
	1	4	75	11.64	80.30*	167.8	381.6	−213.8	+0.29
	1	4	50	7.20	81.71	255.8	348.2	−92.4	+0.17
	4	4	100	18.42	80.14	337.3			
	4	4	75	13.34	80.30	260.3	333.9	−73.6	+0.10
	4	4	50	13.29	83.16	183.7	275.6	−91.9	+0.20
Amphitrite cirrata	1	3	100	9.22?	81.56	242.7?			
	1	3	75	19.74	81.24	448.2	(247.8)	(+200.4)	(−0.27)
	1	3	50	77.21	83.67	340.7	(209.5)	(+131.2)	(−0.13)
	2	5	100	40.62	81.83	337.6			
	2	5	75	11.04	85.03	180.2	267.7	−87.5	+0.21
	2	5	50	49.18	84.28	228.0	283.6	−55.6	+0.15

TABLE I—(*Continued*)

Species	Days of expt.	No. worms	% sea water	Coelomic fluid	Body wall				
				AAN mg./100 ml.	% water	Obs. AAN mg./100 g. H_2O	Exp. AAN mg./100 g. H_2O	"Active" ΔAAN mg./100 g. H_2O	Calc. Δ°C. from total ΔAAN
Neoamphitrite robusta	1	5	100	32.91	78.73	513.8			
	1	6	75	20.57	78.47	489.2	521.8	−32.6	+0.03
	1	6	50	17.29	83.53	313.5	375.0	−61.5	+0.27
	2	7	100	22.61	78.69	587.8			
	2	8	75	27.90	81.53	420.1	491.7	−71.6	+0.22
	2	7	50	63.69	85.54	275.8	366.9	−91.1	+0.41
Thelepus crispus	1	2	100	162.13	71.49	686.2			
	1	2	75	39.38	76.19	484.9	537.8	−52.9	+0.27
	1	2	50	31.03	79.46	409.2	444.8	−35.6	+0.37
	2	5	100	150.63	78.43	516.3			
	2	1	75	44.98	80.50	480.4	454.7	+25.7	+0.05
	2	3	50	90.06	80.20	252.8	463.4	−210.6	+0.35
Aphrodite japonica	2	1	100	0.50	78.48	186.3			
	2	1	75	0.90	83.99	133.8	129.5	+4.3	+0.07
	1	1	50	15.28	87.07	90.9	100.9	−10.0	+0.07
	2	1	50	—	85.01	130.2	119.8	+10.4	+0.13
	2	1	50	~10.12	86.35	86.6	107.4	−20.8	+0.13
Travisia pupa	1	1	100	—	81.30	398.4			
	1	1	75	3.23	82.86	471.7	358.3	+113.4	−0.10
	1	1	50	15.63	84.80	373.6	310.5	+63.1	+0.03
	2	1	100	7.05	78.26	529.9			
	2	1	75	7.09	81.82	556.0	423.9	+132.1	−0.03
	2	1	50	38.97	84.32	311.8	354.7	−42.9	+0.29

* Per cent tissue water not estimated; values for 4-day samples substituted.

decrease in proportion to the fall in amino nitrogen, but the data are insufficient to conclude anything about the possible role of amino-acid degradation during osmotic regulation in any of the animals studied, and so the values have not been included here. It will be seen that in both 75% and 50% sea water the body-wall tissues become more hydrated in almost all species, the single exception being *Nephtys ciliata* where the amount of hydration is marginal or nil. The actual amount of water entering a tissue to account for an increase of 8% in the proportion of tissue water (as occurs in several species after 24 hours in 50% sea water) is considerable. The amount of water (grams) in a quantity of hydrated tissue corresponding to a 100-gram wet tissue sample before hydration is equal to:

$$\frac{\%\ \text{DRY WEIGHT (100\% sea water)}}{\%\ \text{DRY WEIGHT (dilute sea water)}} \times \%\ \text{WATER (dilute sea water).}$$

The expected amino-acid level in the tissue due to hydration alone is then obtained by multiplying the original AAN concentration by the original amount of tissue water in 100 grams of tissue and dividing by the value obtained above.

When the concentration of tissue AAN in osmotically stressed animals which would be expected as a result of hydration is compared with that actually observed (Table I) it is seen that the influx of water generally accounts for a high proportion of the fall in AAN. This is particularly well exemplified in the results obtained with *Aphrodite.*

In several species there appears to be a further decrease in tissue AAN during osmotic stress, which is presumably an energy-requiring process. For convenience, this will be called an "active" change in AAN, although this should not be taken to imply that active transport is necessarily involved. In both *Nephtys ciliata* and *Abarenicola pacifica* the "active" fraction contributes between 50 and 100% of the total AAN decrease. It is of interest that in both these species (and especially in *N. ciliata,* as mentioned above) tissue hydration is minimal. Although movement of tissue water from the extracellular to the intracellular compartment may contribute to the apparent "active" change in AAN levels, even in a limiting case, such as would occur if the extracellular compartment of *Nephtys ciliata,* for example, were to decrease from 30% to 0%, the "active" fraction would still account for about half the total change. In *Nereis vexillosa, Eunice biannulata,* and possibly *Nephtys caecoides,* tissue hydration is greater, while the contribution from "active" changes in AAN concentration varies widely between 0 and 60%. The "active" contribution is not particularly well correlated with the overall effect on freezing point calculated from the total change in AAN level (Table I). All three

TABLE II

Comparison of amino-acid levels (AAN) in the body wall of four intertidal polychaetes in 100% and 50% sea water. The total change in AAN and its contribution to the rise in freezing point are also shown

Species	AAN mM/kg. H_2O		ΔAAN mM/kg. H_2O	Calculated Δ°C.
	Sea water concentration			
	100%	50%		
Nereis vexillosa	331.0	172.2	−158.8	+0.29
	347.1	159.7	−187.4	+0.35
	350.9	159.0	−191.9	+0.36
Perinereis cultrifera (calculated from data of Jeuniaux *et al.*, 1961)	359.0	179.7	−179.3	+0.33
Abarenicola pacificia	275.4	182.7	−92.7	+0.17
	240.9	131.2	−109.7	+0.20
Arenicola marina (calculated from data of Duchâteau-Bosson *et al.* 1961)	424.0	180.0	−244.0	+0.45

species of terebellid show some "active" AAN changes, although again these are not well correlated with changes in freezing point.

In *Travisia pupa* there occurs an increase both in tissue hydration, and, in 75% sea water, apparently in AAN levels in the tissues also, although this requires confirmation in more individuals. Despite the thick cuticle of *Travisia* water is able to enter and reach the tissues. The observed increase in AAN is difficult to explain. It is unlikely that there is any increase in AAN level in *Amphitrite*, the results after 1 day's osmotic stress probably being due to the exceptionally low value obtained in both tissue and coelomic fluid for the control in 100% sea water. Although the "active" contribution to the total change in AAN concentration in *Nereis vexillosa* is variable, the degree of tissue hydration and the calculated elevation in freezing point are remarkably constant for this species. The data (Table II) agree well in both respects with those found for *Perinereis cultrifera* after a 50% dilution in sea water (Jeuniaux *et al.*, 1961). In contrast, when the results for *Abarenicola* are compared with those found by Duchâteau-Bosson *et al.* (1961) on *Arenicola marina* (recalculated to the same units; see Table II) it is seen that *Abarenicola* has a lower tissue AAN concentration initially and hence total changes in AAN level in the latter in 50% sea water make only half the contribution to raising the intracellular freezing point (ΔAAN in *Arenicola marina* = 0.4° C.; in *Abarenicola pacifica* = 0.2° C.).

In most species no reproducible changes in coelomic fluid AAN concentration can be correlated with those in the tissues. Both *Aphrodite* and *Travisia*, however, and possibly also *Amphitrite* show increases in coelomic fluid AAN when levels are decreased in the tissues, while *Thelepus* shows the opposite, namely a decrease in AAN simultaneously in coelom and tissues. Thus, if the "active" decrease in intracellular AAN is caused by an outflux of amino acids from cells to body fluid, an increase will occur in the latter if amino-acid breakdown or excretion is limiting, and a decrease will occur if the rate of removal of coelomic AAN is accelerated more than is necessary to cope with the outflux.

DISCUSSION

In studies on the amino-acid levels in animals adapted to 100% sea water (Clark, 1968), considerable variation was observed within any one species which makes interpretation of results after osmotic stress more difficult. Some possible causes of this variation have already been discussed. It is also probable that some of the species studied here did not attain equilibrium within the experimental period, although in the case of *Nereis vexillosa*, at least, equilibrium appears to be reached within 24 hours. Despite these limitations, several interesting points emerge from the data.

When full account is taken of the dilution of intracellular amino acids resulting from tissue hydration, it is seen that most of the polychaetes studied show only a limited ability to lower their tissue AAN levels further by an "active" process during osmotic stress, at least during the first few days. *Nephtys ciliata* and *Abarenicola pacifica*, both primarily intertidal species, appear to function most efficiently in this respect, whereas subtidal species such as *Aphrodite japonica* and *Travisia pupa* are least proficient. The data suggest that some moderately euryhaline species, as for example *Nereis vexillosa,* are able to withstand considerable tissue hydration and this, rather than the ability to rapidly equilibrate intracellular osmotic pressure, may be vital to their survival in dilute sea water.

Osmoregulation involves exchanges between three fluid compartments: the external medium, the body fluids and the intracellular water. Two physiological boundaries are involved, that between the body fluid and the exterior, comprising both body wall and excretory organ, and the cell membranes separating intracellular water from body fluid. During osmotic stress, euryhaline animals prevent excessive changes in tissue hydration either by maintaining a high osmotic pressure in the extracellular compartment or by reducing the osmotic activity intracellularly, or by a combination of these. Although the intracellular and extracellular compartments are generally thought to be in osmotic equilibrium (Shaw, 1958) the proportions in each of the various osmotically active components usually differ considerably. Thus the total osmoregulatory ability of an animal depends upon the amount and rate of change of all members of the spectrum of dissolved substances in both compartments, and the nature of the compounds exchanged with the environment, together with the rate of water penetration (Smith, 1964) and its consequent effect on the total osmotic pressure in both internal fluid compartments. The variability in the contribution of the "active" AAN component to the total decrease in tissue AAN, as particularly shown in *Nereis* and *Eunice,* suggests that the rates of water movement and amino-acid movement may not be closely synchronized.

In those cases where an "active" decrease in tissue AAN does occur, we may ask what is the fate of these amino acids. Reversible peptide or protein synthesis may occur. Although these appear unlikely mechanisms in certain crustaceans (Shaw, 1958; Florkin *et al.,* 1964), in the ophiuroid *Ophiactis arenosa* osmotic dilution stimulates the incorporation of free amino acids into the alcohol-insoluble fraction, and this is correlated with a decrease in the concentration of free amino acids (Stephens and Virkar, 1966). Reversible synthesis and degradation of amino acids has also been suggested (Allen, 1961; Gilles and Schoffeniels, 1964), the latter authors arguing that the mechanism is controlled by an ion-dependent enzyme system (Schoffeniels and Gilles, 1963; Schoffeniels, 1964a, 1964b). In the present study the changes in ammonia nitrogen levels were too small and too variable to give any clear indication as to whether an increased rate of amino-acid breakdown regularly occurs in polychaete tissues during osmotic dilution.

Another possibility is that amino acids are transferred intact between body-wall tissue cells and coelomic fluid. This is suggested in the present study in those cases where there is a marked increase in free amino acids in the coelomic fluid during osmotic stress, as in *Travisia, Aphrodite,* and possibly *Amphitrite.* This increased concentration occurred despite a probable increase in total volume of coelomic fluid. An increase in coelomic fluid AAN during osmotic stress has also been observed in *Mytilus edulis* by both Potts (1958) and Bricteux-Grégoire *et al.* (1964). If this is also an important mechanism in those other polychaete species where the coelomic fluid amino-acid level remains more or less constant, as in *Nereis* or *Neoamphitrite,* or decreases, as in *Thelepus,* then amino acids must be removed to the exterior at an equivalent or faster rate, respectively.

Among the polychaetes examined, only the terebellids have large numbers of free coelomocytes, and in all three species, these cells comprise about half the volume of the coelomic fluid. It is therefore possible that osmotic-stress-induced changes in AAN levels in the non-cellular coelomic fluid fraction in these worms are the result of amino-acid movement into or out of the coelomocytes, which themselves probably contain AAN-levels comparable to those in cells of the body wall. However, only in *Thelepus*, where there is a marked fall in coelomic-fluid AAN, is it likely that the coelomocytes could be playing a role in removal of free amino acids from the coelom, either by degrading them or assimilating them into an osmotically less active form, such as peptides or proteins.

The boundary between body fluid and the exterior, be it composed of body wall, or of specialized excretory organ, or both, constitutes another site actively involved in osmotic adjustment. It is known that free amino acids are taken up across the body wall by marine annelids (Stephens and Schinske, 1961; Stephens, 1963, 1964) and sipunculids (Virkar, 1963) and that the rate is correlated with external salinity (Stephens, 1964). This suggests a relation between Na^+ concentration and active amino-acid transport across the body wall although some uptake at appropriate external osmotic pressures but in the absence of external Na^+ does occur (Stephens, 1964). In a case such as *Thelepus*, where coelomic fluid amino-acid levels fall by an amount more than proportional to the external dilution, an energy-utilizing process is indicated, and the possibility of extrusion of unchanged amino acids across the body wall by the reverse process of active uptake cannot be ignored. Although ammonia is the primary nitrogenous excretory product of some animals, even during osmotic stress (Needham, 1957; Florkin *et al.*, 1964), large amounts of free amino acids are excreted by many types of planktonic animals (Johannes and Webb, 1965). This might also occur in other groups which, like the polychaetes, have not yet been studied in this respect.

The present data can only suggest possible directions for future detailed studies, but they indicate that polychaetes, even closely related species, are likely to regulate their amino-acid levels under osmotic stress by a variety of mechanisms.

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SUMMARY

1. Ten species of polychaetes were exposed to 75% and 50% sea water for 1 to 4 days, and measurements of amino-acid levels in the coelomic fluid and body wall were made. Individuals of all species, both intertidal and subtidal, survived the experimental period at both dilutions.

2. Osmotic stress at lowered salinities results in a decrease in intracellular amino-acid levels. This is primarily due to tissue hydration, although in some species, especially *Nephtys ciliata* and *Abarenicola pacifica*, hydration is minimal or absent and "active" movement of amino acids contributes a larger share of the decrease.

3. In several species osmotic dilution causes a rise in coelomic fluid amino-acid levels, suggesting direct transfer of amino acids from tissues to body fluid. In one species, *Thelepus crispus*, there is a 50 to 75% fall in coelomic fluid amino-acid level, although this is not true in two closely related species. The data indicate that polychaetes may use a variety of mechanisms to regulate their amino-acid levels

during osmotic stress. It remains to be seen whether the distribution of regulative mechanisms among polychaete species can be correlated either with type of habitat or with phylogenetic relationships.

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PERCEPTION OF EXTERNAL OXYGEN BY THE BURROWING SHRIMP, CALLIANASSA CALIFORNIENSIS DANA AND C. AFFINIS DANA[1]

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Ventilative behavior during oxygen stress has been described in many animal species (Prosser, 1961), but little is known about the neural control of these responses. This is especially true in aquatic animals, where oxygen lack rather than carbon dioxide is considered critical for ventilation. Except for the mammalian carotid body chemoreceptors (Eyzaguirre and Koyano, 1965), oxygen perception is still virtually an unexplored area.

An internal oxygen receptor was hypothesized for the crayfish (Larimer, 1964) to explain a transitory scaphognathite inhibition which occurred with resumption of heart activity after a period of drug-induced cardiac arrest. The possible action of other blood-borne agents was not eliminated, however, and the response has yet to be shown dependent on blood oxygen levels.

More substantial evidence for oxygen perception was obtained in *Limulus* (Waterman and Travis, 1953) where anoxic ventilatory arrest was abruptly terminated with application of aerated sea water. The receptor site is still unknown, though response latencies suggested an external location.

The present experiments were undertaken with the possibility that *C. californiensis* and *C. affinis* might be particularly useful for studying oxygen-sensing mechanisms. On the West Coast of North America they live in burrows in muddy, intertidal areas, potentially oxygen-poor habitats where sensitive respiratory chemoreceptors would seem advantageous. A respiratory function has been proposed for sustained pleopod beating in tubes and burrows (MacGinitie, 1934) and we now demonstrate sensory mechanisms enabling these animals to monitor external oxygen concentrations and make appropriate ventilating responses.

MATERIALS AND METHODS

Specimens of *C. californiensis* were dug from mud flats at Elkhorn Slough and Point Mugu in California, and specimens of *C. affinis* were obtained from a rocky point near Goleta, California. Animals were kept either in glass tubes or mud-filled aquaria with adjustable panes for observation of activity in the burrows. Experiments were done in an air-conditioned room (approx. 20° C.) with sea water taps available.

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Both species have three paddle-like pleopods well adapted for swimming and moving water. Pleopod beating was always sufficiently slow (120/min. or less) to enable visual monitoring, with each stroke registered on an event marker. The unrestrained animal could thereby move about during experiments, in a tube approximating actual burrow conditions. All pleopod activity was recorded without distinguishing between swimming and ventilatory strokes, since relatively little swimming occurs in tubes and burrows.

Low-oxygen sea water (10 mm. Hg or less) was obtained by bubbling nitrogen through sea water as it passed down a glass column (Fry, 1951) 1 m. tall and 5 cm. in diameter, filled with glass beads. Effluent pO_2 was determined with the Standard Macro Winkler Method (Barnes, 1959) and a Beckman oxygen electrode calibrated with 5% oxygen. Electrode readings were registered beside event marks indicating pleopod strokes. Animal chambers and test solutions used in these experiments were kept continuously immersed in flowing sea water baths (20° C.) to eliminate temperature discrepancies.

RESULTS

To observe the ventilative responses of *C. affinis* in low pO_2, an animal was placed in a plastic gauze (5 mesh/cm.) tube 20 cm. long and 13–26 mm. in diameter. This was inserted into a glass chamber 20 cm. long and 26 mm. in diameter. A rubber stopper at one end supported the oxygen electrode and a small intake tube for sea water. A sea water exit tube was provided at the opposite end. Animal responses in plastic gauze tubes of various diameter could thus be compared, without altering sea water movement inside the glass chamber. Tap sea water was passed through the chamber (20–30 ml./min.) for 15 minutes, then low-oxygen sea water at the same rate for 30 minutes, followed by tap sea water again for 15 minutes.

Some pleopod beating usually occurred at low pO_2, but if the diameter of the plastic gauze tube (26 mm., Fig. 1A) was larger than the width of the animals (uropod span, 13–24 mm.), there was relatively little response.

More ventilation occurred (Fig. 1B) when the animals were placed in plastic gauze tubes 13–20 mm. in diameter. This difference was clearly not a result of learning or accumulated anoxia, for a diminished response (as in Fig. 1A) was again obtained if the animal was returned to the 26-mm. tube and the experiment repeated for the third time.

The mean stroke frequency for 5 animals in the 26-mm. tube during the 30-minute period when low-oxygen sea water was flowing (Fig. 1, between arrows) was 2/min. ± 0.62 (standard error). For the same 5 animals in 13–20 mm. tubes, the mean frequency was 22/min. ± 5.20. Analysis of variance indicated p was less than 0.01, clearly showing that tube diameter must be considered when planning ventilation experiments with these animals. During sustained ventilation they often lie upside down and/or brace their walking legs against the walls of their burrows, and apparently this contact is important for ventilation to occur.

The mean ventilation response threshold in this experiment was 28 mm. Hg, as measured with the oxygen electrode. To avoid disturbing the animals, sea water mixing in the chamber was not attempted, but substantial mixing probably resulted from animal movements.

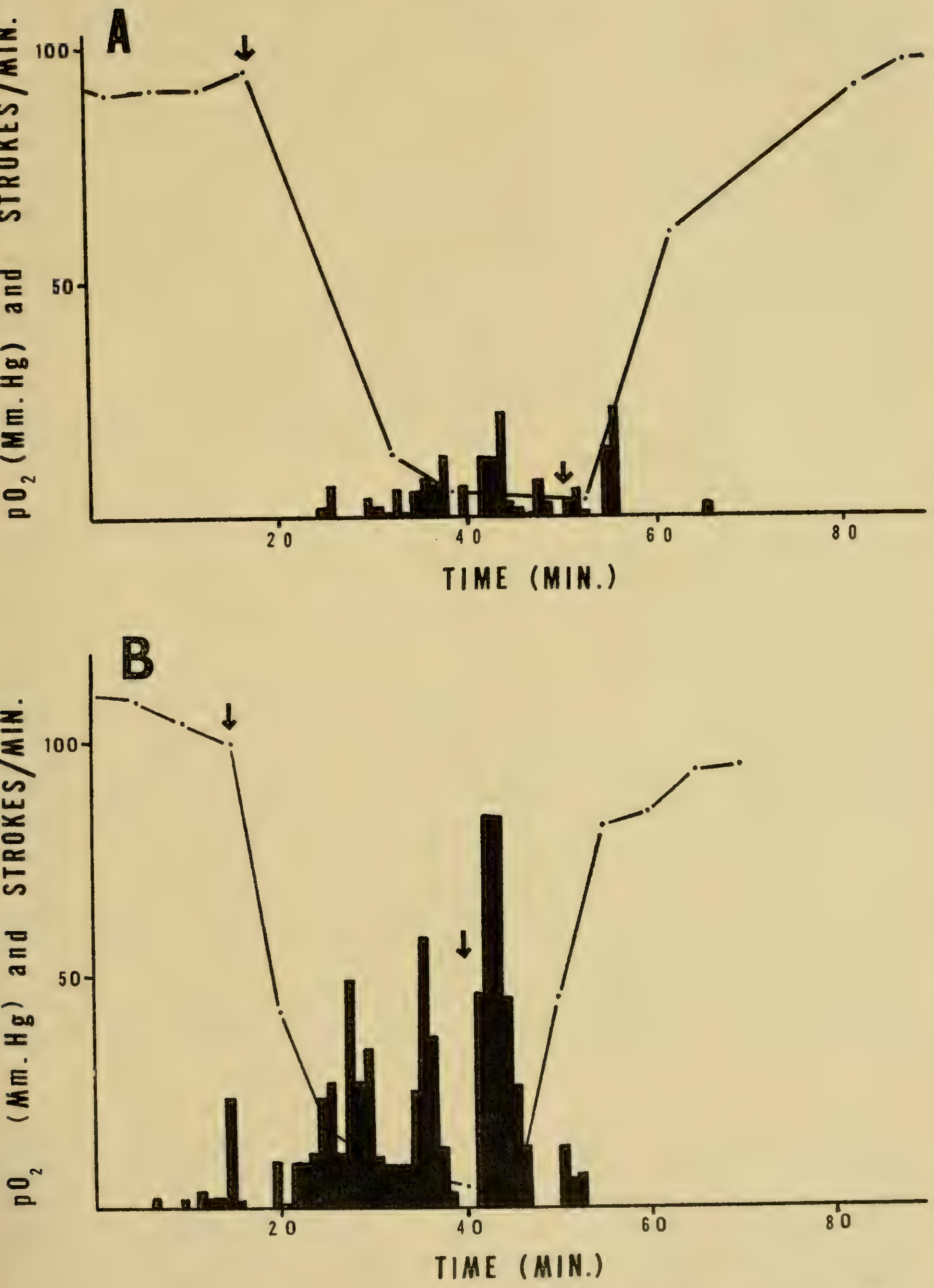

FIGURE 1. Relationship between pleopod strokes/min. and sea water pO_2 for *C. affinis* in a 26-mm. (A) and a 13-mm. (B) diameter tube. Arrows indicate admission of low-oxygen and tap sea water, respectively.

Ventilation sometimes increased markedly when tap sea water was readmitted into the chamber (Fig. 1B, second arrow). This response was most striking if the animal was placed with its head toward the intake end so that pleopod power strokes (retraction) had the apparent effect of bringing sea water with oxygen into the chamber. These responses to environmental pO_2 suggested the presence of an oxygen receptor and prompted a further test of such perception in *C. californiensis.*

This species is easily obtained in mud-flats, their burrows are accessible and pO_2 regulative behavior seemed likely since burrow openings are exposed for hours at low tide. During low tide at Elkhorn Slough, Winkler analysis (Barnes, 1959) of water aspirated from 20 burrows of *C. californiensis* indicated pO_2 less than 20 mm. Hg. Under these conditions the ability to detect aerated sea water and ventilate as in Figure 1B, second arrow, would be particularly advantageous during the incoming tide as burrow openings are again covered with sea water. The experiment was arranged to approximate these burrow conditions.

C. californiensis was placed in a glass tube 60 cm. long and 17 or 20 mm. in diameter, sizes found to promote ventilation. Low-oxygen sea water flowed through the tube (50 ml./min.) for 30 min., with the animal's head toward the intake. The animal explored, ventilated and eventually became relatively quiescent near the rear of the tube. Flow was stopped and after one minute 5 cc. of aerated or low-oxygen sea water were injected with a syringe into the intake end. Apparently in response to water movement during injection, the animal moved forward. Pleopod activity was recorded during the 1–5-min. period while the animal remained at the intake half of the tube.

If aerated sea water had been injected, the animals began vigorous ventilation within a few seconds after reaching the region of aerated sea water, about 10 cm. from the intake end of the tube. Pleopod activity eventually stopped, the animal withdrew, and low-oxygen sea water was passed through the tube for 5 minutes before another test was made. When 5 cc. of low-oxygen sea water were injected, again the animals moved to the intake end, but there was relatively little ventilation.

Five repetitions of the experiment were done with each of 5 animals. The mean number of pleopod strokes following injection of low-oxygen sea water was 14 ± 7.78 (standard error) and 77 ± 14.48 after aerated sea water. Analysis of variance indicated p less than 0.01, showing the animals could detect and respond differentially to external oxygen concentrations.

The pO_2 detected in the above experiment was calculated to be about 50 mm. Hg, using a dilution factor obtained by a photoelectric dye dilution method (Case, 1962).

Discussion

Varied responses to respiratory stress have been reported for crustaceans (Prosser, 1961), and it is clear from these experiments that *Callianassa* must be included among those animals which hyperventilate in low pO_2. Walshe-Maetz (1952) suggested such regulative capacity is related to habitat, since brackish-water animals like *Gammarus* hyperventilate with low oxygen, while ventilation rates in marine and semi-terrestrial species are less likely to be affected. Certainly *Callianassa* is no exception to this rule.

The pleopod power stroke in *Callianassa* is capable of moving the animal forward, or when station is maintained, water can be propelled caudally. The difference in ventilative behavior observed in large and small tubes (Fig. 1) probably reflects a difference in the ability of the animal to counter the forward thrust of pleopod strokes. Body slippage, and consequent loss of pumping efficiency, would seem most easily prevented in smaller size tubes where maximum body surface can contact tube walls.

The demonstration in these experiments that *Callianassa* can detect external oxygen concentrations is clearly of adaptive significance. Animals during low tide can commonly be dug from burrows nearly a meter beneath the surface. The ability to move toward burrow openings, detect oxygenated sea water and hyperventilate with the incoming tide would reduce the period of anoxia most likely to occur during low tide. MacGinitie (1927) has reported that *Callianassa* was easier to dig near the beginning of the receding tide and as the water was just returning to the burrows, because the animals were nearer the surface at these times.

The location of the oxygen receptor in *Callianassa* is still unknown, but preliminary experiments with antennal ablation failed to abolish the response. Further localization experiments are presently planned.

SUMMARY

1. Pleopod ventilative responses in low-oxygen sea water were investigated in two species of *Callianassa*, with animals relatively unrestrained during experiments, in tubes simulating actual burrow conditions.

2. In *C. affinis* hyperventilation stroke frequencies up to 120/min. were recorded in low pO_2, with maximum ventilation oftentimes occurring upon readmission and detection of oxygen-containing sea water.

3. In low-oxygen sea water *C. californiensis* was clearly able to discriminate between samples of aerated and low-oxygen sea water, as indicated by significantly greater ventilation beginning within a few seconds after detecting the oxygen-containing sea water.

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SIALIC ACID IN EGG CAPSULES AND OVIDUCTS OF THE NEWT, NOTOPHTHALMUS VIRIDESCENS[1]

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The deposited ovum of the newt, *Notophthalmus viridescens,* is enclosed in a capsule composed of five structurally and cytochemically different layers of "jelly," designated layers J1 through J5, from inner to outer (Humphries, 1966). The layers making up the capsule are successively deposited about the egg by five rather distinct zones of the oviducts, designated regions "A" through "E," from anterior to posterior (Humphries and Hughes, 1959; Humphries, 1966). The histochemical reactions of the secretory epithelium of each of the five secreting regions of the oviduct correspond to those of the jelly membranes which they secrete. The marked structural and cytochemical differences in the layers of the capsule have stimulated interest in several problems relating to their functions. Among these is the problem of the role of "jelly" in fertilization. Shaver (1966) and Metz (1967) have recently reviewed the subject for the Amphibia.

One of the compounds of interest with regard to fertilization is sialic acid. Warren (1960) reported that considerable amounts of sialic acid are released from eggs of the trout when they are fertilized or artificially activated, but the significance of his observation is unknown. Soupart and Noyes (1964) gave cytochemical evidence for the presence of sialic acid in the zona pellucida of various mammalian eggs and suggested that sialic acid residues may play a role in sperm penetration. Later work by Soupart and Clewe (1965), in which they demonstrated an inhibition of sperm penetration after treatment of rabbit eggs with neuraminidase, gave support to the suggestion.

Little is known about the occurrence or function of sialic acids in amphibian eggs or their membranes. The earliest report on the subject is the statement of Warren (1963) that sialic acid is present in frog eggs (*Rana catesbeiana*). Warren did not specify whether he was referring to the egg plus its capsule, or to the egg alone. Cytochemical evidence for the presence of sialic acid in the egg jelly of *Notophthalmus viridescens* was reported by Humphries (1966), followed by a preliminary report (Humphries and Workman, 1966) of the demonstration of sialic acid in oviducts and capsular jelly through use of the thiobarbituric acid assay procedure of Warren (1959). Sialopolysaccharides have been discovered in the egg mantle of *Rana latastei* and *Bufo vulgaris* (Bolognani *et al.,* 1966) and sialic acid has been reported in the egg jelly of *Xenopus laevis* (Freeman, 1967) and *Rana pipiens* (Lee, 1967).

The cytochemical evidence for the presence and distribution of sialic acid in egg membranes of *Notophthalmus viridescens* (Humphries, 1966) is not conclu-

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sive; therefore this study was undertaken primarily to establish whether sialic acid is indeed present in the egg capsules and oviducts of this species, and, if it is present, to gain more definite information as to which layers of the capsule contain it. We also desired to determine whether there is sialic acid in the egg itself, either before or after its passage through the oviduct.

Materials and Methods

Adult *Notophthalmus (Diemictylus, Triturus) viridescens* females were obtained from the vicinity of Franklin, North Carolina, and maintained in greenhouse tanks and/or a refrigerator at 12° C. until used. All animals were in breeding condition. Most of the animals received two intracoelomic injections of 150 units of chorionic gonadotropin (Antuitrin "S," Parke, Davis) for the induction of ovulation. Those animals which received no gonadotropin were apparently no different from the injected animals in the sialic acid content of their oviducts; therefore the information from injected and non-injected animals is combined. All eggs were obtained from females which had received hormone treatment.

The thiobarbituric acid procedure of Warren (1959, 1960) was adapted to our material and used for assays of sialic acid. After decapitation of the animals, segments of each region (A through E) of the oviduct were removed and homogenized in a glass homogenizer in 3 to 5 ml. of cold 0.1 *M* KCl. Transitional areas between oviducal regions were not used, since we desired to minimize possible contamination between adjacent regions. Aliquots of the homogenate were usually taken for determination of both free and total sialic acid content and for dry weight determination. Studies were made on oviducts of twelve females, but in some cases the readings obtained from a sample were outside the range of the method, and these are not reported. Also, dry weight determinations were not made in every case. The usual procedure was to use the oviducts of a single animal for each assay, but in one instance the assay was performed on pooled oviducal segments from four animals.

Eggs were assayed similarly, ordinarily using homogenates of fewer than 15 eggs in 2 to 3 ml. of 0.1 *M* KCl. In one instance, however, 58 coelomic eggs from a single animal were used. Region E eggs were removed from the oviducts and assayed directly or decapsulated with watchmaker forceps for separate assay of eggs and capsules. Assays were made, whenever possible, of coelomic and oviducal eggs from the same animal. Twelve animals were used as sources of eggs, but not all animals were used for each type of assay. Since only small amounts of material were available, the results of the assay of eggs and capsules are reported as amount of sialic acid per egg or per jelly capsule, rather than in terms of the amount per unit dry weight. Readings were made in a Zeiss PMQ II Spectrophotometer. Equation 2 of Warren (1959) was used to calculate the concentration of sialic acid. Standards, consisting of N-acetylneuraminic acid (Sigma Chemical Co.) in amounts of 0.01, 0.03, and 0.05 μmole, were also used. The molecular weight of N-acetylneuraminic acid (309) was used for all calculations. Student's t-test was used as a test of statistical significance.

According to Warren (1959), the production of color in the thiobarbituric acid assay varies linearly with concentration of N-acetylneuraminic acid in the range

TABLE I

Sialic acid in oviducal regions (mg./g. dry weight)

	Region A	Region B	Region C	Region D	Region E
Total (mean ± SE)	*	94.03 ± 19.71	*	121.08 ± 15.80	*
Free (mean ± SE)	*	*	*	24.56 ± 2.96	*

* None was detected. See, however, the explanatory statement in "Materials and Methods."

0.01 to 0.06 μmole. For several materials (*e.g.,* region E) it was not possible to obtain assays which approached the 0.01 μmole level, even in homogenates containing a markedly higher concentration of tissue (or eggs) than was adequate for the demonstration of sialic acid in materials in which positive results were readily obtained (*e.g.,* region D). Therefore, when we state that sialic acid was not found in a given material, we mean that we did not obtain values within the usable range. Such a statement should not be taken to mean that "absence" of sialic acid has been demonstrated.

RESULTS

Sialic acid was always found in regions B and D of the oviducts, but never in regions A or E (Table I). In a single instance a positive finding was obtained from a homogenate of region C, but we believe this to be due to accidental contamination with a portion of region B. Values for total sialic acid content in regions B and D are not significantly different, but the two regions differ in that a part of the total sialic acid content in D is free, while free sialic acid was not detected in B.

A summary of results obtained from the assay of oocytes and jelly capsules is presented in Table II. Sialic acid was not detected in coelomic oocytes or in decapsulated oocytes taken from region E. Assay of oocytes from region E (with intact jelly capsules) and jelly capsules alone yielded values which do not differ significantly. In addition, for any one animal, values for encapsulated eggs and isolated capsules were similar (Table III), but there was considerable variation between the encapsulated eggs (or capsules) of different animals.

TABLE II

Free and total sialic acid in coelomic eggs, decapsulated Region E eggs, encapsulated Region E eggs and isolated egg capsules

	(μg. per egg or capsule)	
	Total	Free
Coelomic eggs	*	*
Decapsulated eggs	*	*
Encapsulated eggs (mean ± SE)	35.1 ± 3.8	4.9 ± 1.1
Capsules (mean ± SE)	26.8 ± 5.3	9.2 ± 3.5

* None was detected. See, however, the explanatory statement in "Materials and Methods."

Discussion

The striking differences in the various regions of the oviduct, previously reported by several investigators (Adams, 1940, 1950; Humphries and Hughes, 1959; Kambara, 1956a, 1956b, 1957a, 1957b; Lebrun, 1891; von Wahlert, 1953) are again demonstrated in the results reported here. Clearly, regions B and D contain sialic acid, and in rather high concentration. The difference between the two regions with regard to "free" sialic acid is interesting, but should not be accepted without reservation, since Eichberg and Karnovsky (1966) have shown that the Warren procedure itself may give rise to artifactual "free" sialic acid. Nevertheless, it appears obvious that the sialic acids of the two regions differ somewhat, perhaps in their form, linkage, or combination with macromolecules. The possibility that other parts of the oviduct contain some sialic acid cannot be eliminated, and, judging from Warren's (1959, 1963) study of the distribution of sialic acid in vertebrate tissues, it seems likely that at least small amounts are present throughout the oviduct.

Table III

Total sialic acid in encapsulated Region E eggs and in egg capsules isolated from eggs of the same animal

Animal number	Encapsulated eggs (μg./egg or capsule)	Capsules (μg./egg or capsule)
712	25.7	25.6
67	13.9	8.3
621	37.2	36.1
810	19.9	19.8
811	43.4	35.9

Since regions B and D secrete jelly layers J2 and J4, respectively, these layers probably contain sialic acid. Cytochemical evidence obtained by one of us (Humphries, 1966) indicated that layers J2 and J5 contain sialic acid but did not exclude the possibility of its presence in other layers. The present results, then, are in only partial agreement with expectation. The available cytochemical methods are known to be of limited usefulness, however, and the present findings provide a firmer basis for conclusions. It is conceivable, on the other hand, that an unbound sialic acid might move from one layer to another, such as from J4 into J5, resulting in a positive cytochemical test in J5.

In view of the fact that we were unable to obtain any evidence of the presence of sialic acid in either coelomic oocytes or in oviducal oocytes divested of their jelly capsules, it appears that there is little or none in the egg itself. There is good agreement between values obtained for total sialic acid content of intact region E eggs (encapsulated) and those for isolated capsules from other eggs of the same animal. Furthermore, pooled data from separate determinations on material from different animals show no significant difference between the sialic acid content of isolated jelly capsules and region E eggs plus their capsules. Therefore all, or nearly all, of the sialic acid of the mature egg with jelly envelopes appears to be in the envelopes. The techniques used here, however, do not exclude the possibility that small amounts of sialic acid may be associated with the oocyte itself or with the vitelline membrane—either prior to or following the passage of the egg through the oviduct.

The significance of the presence of sialic acid in the gelatinous coverings of the egg is unknown. Of several functions which might be suggested, the possible role of sialic acid in fertilization is most intriguing. It is generally agreed that in the Amphibia at least some "jelly" is usually necessary for sperm penetration (see Humphries, 1966, for references), but little is known as to exactly what role(s) the jelly has in the process. The evidence advanced by Soupart and Noyes (1964) and Soupart and Clewe (1965) suggests that sialic acid is involved in the fertilization of mammalian eggs, and the findings of Warren (1960) imply that sialic acid is associated in some way with the events of fertilization of trout eggs. Extensive observations by McLaughlin (1967) on sperm penetration in eggs of *Notophthalmus viridescens* are also suggestive. She found that artificially inseminated eggs from region B showed a significantly higher percentage of sperm penetration than eggs from region A. Similarly, those from region D showed a significantly higher percentage of sperm penetration than those from region C, but there was no significant difference between eggs from regions B and C. Sperm penetration was significantly lower in eggs from region E than in those from region D. Other observations by McLaughlin seemed to show that layer J4 is particularly important with regard to facilitation of sperm passage through the capsule. These findings, therefore, seem to indicate that layers J2 and J4, which apparently contain sialic acid, have a special significance for sperm penetration. At the present time, however, the available information concerning a relationship between sialic acid and fertilization is sufficient only to excite speculation and stimulate additional study of the problem.

SUMMARY

1. The occurrence of sialic acid in eggs and oviducts of *Triturus viridescens* has been studied.

2. Regions B and D of the oviducts contain sialic acid. A part of the sialic acid in D may be free. Sialic acid was not detected in regions A, C, or E.

3. Sialic acid is present in the egg capsule, but was not detected in coelomic eggs or in decapsulated eggs from oviducal region E. Part of the sialic acid of the capsule may be free.

4. Available evidence suggests that the sialic acid of the capsule is in layers J2 and J4, and perhaps in layer J5, as well.

5. The possible significance of the findings with regard to fertilization is discussed.

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ON THE MORPHOLOGY, FEEDING, AND BEHAVIOR OF MAGELONA SP.

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Although the genus *Magelona* has been recognized for more than one hundred years, our knowledge of the internal anatomy of this polychaete is based mainly on the work of one investigator. This is due, for the most part, to the relatively small size of most magelonids and to the fact that most biologists are unaware of the presence of this most interesting genus in the vicinity of many marine laboratories over the world.

In his brief original description of the genus, F. Müller (1858) mentioned a number of unique morphological characters which, even then, served to set *Magelona* apart from other polychaetes, *i.e.,* papillated tentacles, an anterior region of nine segments, a pale, violet, corpusculated blood, and paired segmental lateral vessels with pulsatile vesicles in the posterior region.

McIntosh contributed nearly all of the anatomical information on *Magelona* in a preliminary note (1877), an exhaustively detailed and illustrated account in German (1878), and unillustrated English translations of the latter (1879 and 1911), all of which formed the basis of the account of *Magelona* in his monograph of British Annelids (1915).

Most subsequent workers who have dealt with the genus have restricted their observations to such features of external anatomy as were necessary to define new species or to confirm previously known species. Mésnil (1896) included a few observations on the circulatory system which are of interest and Jones (1963) reported the presence of ciliated lateral organs and, apparently secretory, dermal inclusions. Romieu (1923) observed that the entire somatic musculature of *Magelona* is singly or doubly striated and pointed out that the longitudinal muscles closely resemble those of *Ascaris megalocephala.* Orrhage (1962) confirmed Romieu's observation of "Nematoden-Typus" muscle cells in *Magelona,* as well as in a number of other polychaetes. More recently, Orrhage (1966) has reported on the anatomy of the central nervous system of *Magelona* in his consideration of the central nervous systems of the sedentary polychaetes.

There are a number of inconsistencies between the present account and that given by McIntosh in his various papers on the species identified as *Magelona papillicornis.* To some extent, these discrepancies may be due to specific differences, but probably many of the differences are due to differences in observational techniques. Where most of the present studies were made on critically prepared histological material with high quality optical equipment, McIntosh's observations were made on free-hand sections. It is indeed a tribute to McIntosh's powers of observation that he was able to carry out such extensive studies with as few misinterpretations as are present.

In spite of what would appear to be an overall thorough treatment of the morphology of *Magelona*, especially through the work of McIntosh, recent access to large numbers of living specimens of this genus has brought to light a number of interesting additions and corrections to previous observations.

MATERIALS AND METHODS

The specimens of *Magelona* sp. utilized in this study were obtained in July and August, 1966, from the Buzzards Bay end of Northwest Gutter, between Naushon and Uncatena Islands (lat. 41° 30.9′ N; long. 70° 42.7′ W) near Woods Hole, Massachusetts. In August, 1966, and January, April, July, and August, 1967, they were also collected at Stony Beach [= M.B.L. Beach] at Woods Hole (lat. 41° 31.8′ N; long. 70° 40.5′ W). In both localities the substrate in which the worms were found was a well-sorted fine sand ($< 0.005\%$ of a substrate sample, by weight, was retained by a number 35 sieve: openings 0.495 mm., 81.7% by a number 60 sieve: openings, 0.246 mm., and 98.8% by a number 120 sieve: openings, 0.124 mm.) at about two to four feet below Mean Low Water.

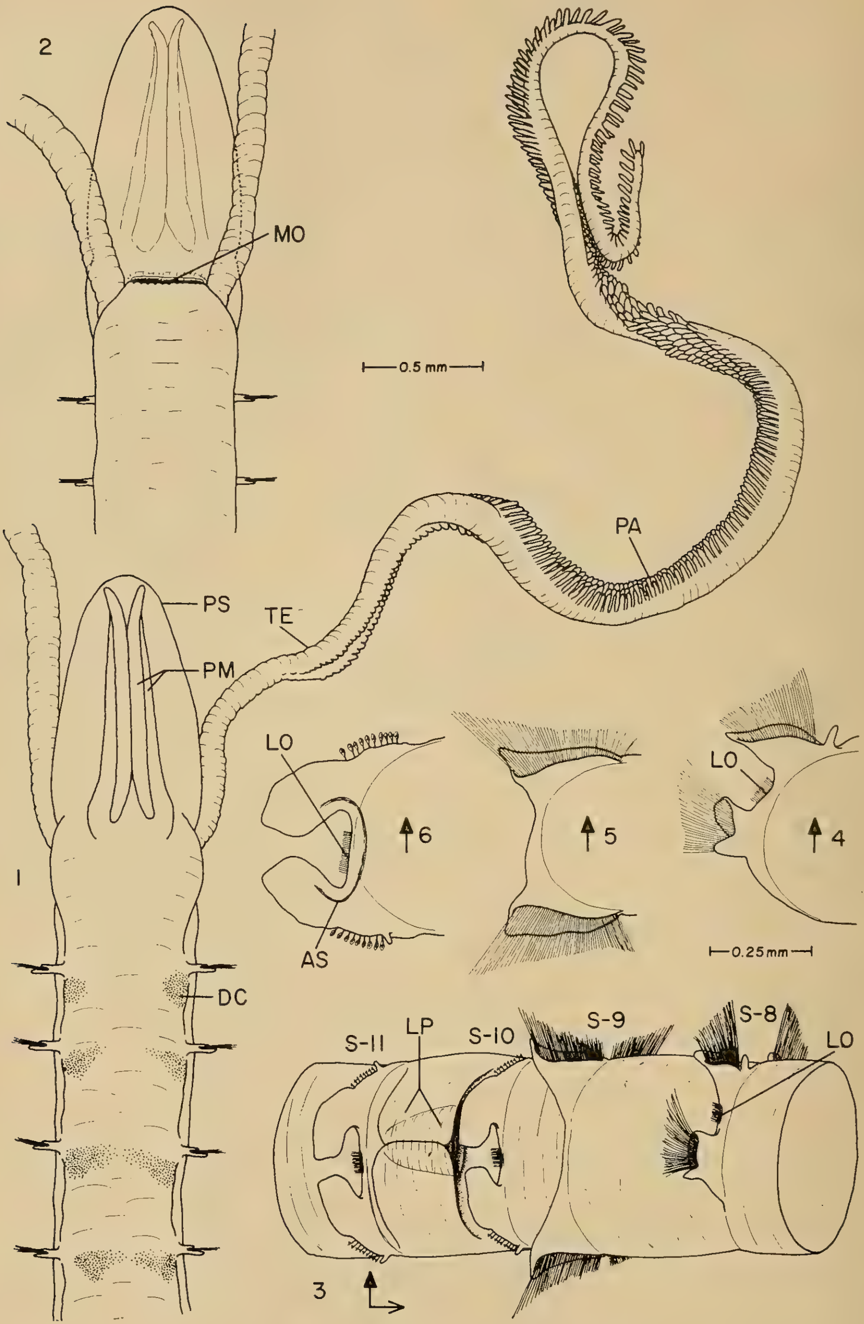
Nearly all animals were adult forms as shown by the presence of eggs or sperm in the posterior regions of most of those collected. Adult forms, relaxed and preserved, were approximately 0.75 mm. in diameter, 30 mm. in length, and were comprised of from 43 to 60 setigerous segments.

Those specimens used for histological examination were relaxed in isotonic magnesium chloride or 0.15% propylene phenoxetol in sea water and were fixed in Bouin's with acetic. They were serially sectioned in paraffin at 5 and 6 microns and stained in Weigert's haematoxylin and eosin, Carson's modification of Mallory's triple stain, and periodic acid-Schiff's reagent with alcian blue, using diastase controls (= PAS/AB).

It will be noted that no specific determination has been offered for the magelonids under study. Although *Magelona rosea* Moore was described from the Woods Hole area (Moore, 1907), the study material does not agree with the description of *M. rosea* and the holotype of this species could not be found in The Philadelphia Academy of Natural Sciences for comparison. The magelonid studied here is more closely related to, but not identical with, the species referred to as *M. papillicornis* Müller by McIntosh (1877, 1878, 1879, 1911, and 1915) and other European workers. Thus, until taxonomic studies presently in progress can be completed, the species must be referred to merely as *Magelona* sp.

EXTERNAL STRUCTURES

All members of the family Magelonidae are unique among the polychaetes for the broad, flattened prostomium (Figs. 1 and 30, PS) which merges posteriorly with the achaetous peristomium. A pair of long slender tentacular palps or tentacles (Fig. 1, TE), bearing papilliform structures on their distal portion, arise from the ventrolateral surface of the prostomium at the level of the mouth opening. The proboscis (Fig. 31, PR) everts as a pouch from the lower part of the mouth area. The anterior eight setigerous segments, all provided with similar setae and similar parapodial lobes or lamellae (Figs. 3, S-8, and 4), comprise an anterior region. The ninth setiger may be similar to the eight preceding setigers (as in the case of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Figures 1–6.

M. rosea, based on its original description) or may be abruptly different (Figs. 3, S-9, and 5) in terms of both parapodial lamellae and setae (as in the case of *Magelona* sp.). The tenth setiger (Figs. 3, S-10, and 6) marks the beginning of a posterior region which, in all known species of *Magelona*, continues to the posterior end; parapodial lamellae, which are supported by internal arcuate setae (Figs. 6 and 29, AS), and setae of this region are similar, and differ from those of the first nine setigers.

On the lateral surface between the tenth and the eleventh setal fascicles, in nearly all specimens, male and female alike, mature and immature, there are paired structures which have been noted in certain other species of *Magelona*. These (Figs. 3 and 10, LP), the "lateral organs" of McIntosh (1879, 1911, and 1915), the "lateral pouched membranes" of Hartman (1961), and the "lateral pouches" of Jones (1963), are open anteriorly and blind-ending posteriorly, with no sign of communication to the internal spaces of the body. The pouches consist of dorsal and ventral flaps overlapping a funnel-like central structure which is attached basally to the body wall. The distal edge of the funnel is fused to the anterior margin of the pair of flaps. The whole lateral pouch is well-supplied with dermal inclusions of the type reported by Jones (1963, p. 29) as well as the so-called "bacillary cells" of McIntosh (1911, p. 426). In addition, the inner surface of the central part of these structures is provided with a thick layer of material which, both in appearance and staining reactions, is similar to the cuticle.

As mentioned above, the lateral pouches are almost invariably present on both sides between setigers ten and eleven. More posteriorly the lateral pouches occur singly and with a certain amount of variability. It would seem that there is a rather loose basic pattern in the appearance of these lateral pouches, in that they occur on every fourth segment and those on one side alternate with those on the

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Key to lettering and symbols: setigerous segments are indicated thusly: S-9 = ninth setigerous segment; arrows with solid heads indicate dorsal and those with open heads indicate anterior; AS, arcuate setae supporting posterior lamellae; AT, afferent tentacular vessel; BC, blood cells; BP, bypass from DV-2 to ventral sinus; CC, coelomic cavity; CL, circular and longitudinal muscles; CM, circular muscle of the gut; CU, cuticle; DC, dermal inclusions; DL, dorsal longitudinal muscle; DO, dorsal outpocketing of the ventral sinus; DR, dorsal retractor muscle; DT, dorsolateral transverse muscle; DV-1, DV-2, DV-3, DV-4, regions of the dorsal vessel (see text); ET, efferent tentacular vessel; GA, giant axon; GE, gut epithelium; GU, gut; IC, intersegmental constriction of body wall; LM, longitudinal muscle; LO, lateral organ; LP, lateral pouch; MC, mucous cells; MO, mouth; MS, mesentery; NH, nephridial tissue; NL, neuropile of ventral nerve cord; NP, nerve plexus; NT, nerve tissue; OC, oöcyte; OM, oblique muscle; PA, papilla(e); PM, prostomial muscular cylindroids; PV, pulsatile vesicle of the segmental loop; PR, proboscis; PS, prostomium; RV, ring-like vessel; SE, septum; SG, supraoesophageal ganglionic area; SF, basal region of a setal fascicle; SL, segmental loop; SV, segmental vessel within septal walls; TE, tentacle; VA, valve; VC, ventral medial longitudinal muscles; VL, ventral lateral longitudinal muscle; VM, vertical muscle; VO, ventral outpocketing of ventral sinus; VR, ventral retractor muscle; VS, ventral sinus; VT, ventral transverse muscle; VV, ventral vessel; WV, wall of ventral sinus.

FIGURE 1. Dorsal view of anterior region of *Magelona* sp.

FIGURE 2. Ventral view of anterior region of *Magelona* sp.

FIGURE 3. Lateral view of setigerous segments 8 through 11, from the right side.

FIGURE 4. Anterior view of eighth right setiger.

FIGURE 5. Anterior view of ninth right setiger.

FIGURE 6. Anterior view of tenth right setiger.

other. This somewhat regular pattern commences at about the level of setigers 20 to 23 and may continue to the posterior end of the body.

The function of the lateral pouches remains unknown. It would not seem to be related to reproduction, for these structures are found in males, females, and juveniles. The inner surface of the pouches is provided with a thick layer of apparently impermeable cuticle, not the type of surface one would associate with secretory activity. Finally, the lack of any communication with the interior of the body at the inner end of the pouches precludes the possibility of inward or outward flow of external materials or body fluids, respectively.

Lateral Organs

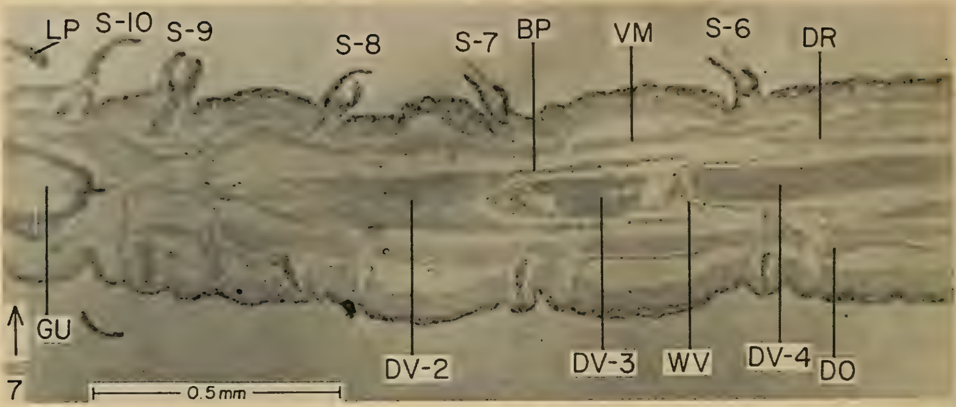
Lateral organs, *i.e.,* ciliated sensory structures, segmentally arranged on the lateral surface of the body wall of a number of sedentary polychaetes, have been reported upon by Rullier (1951). In this paper Rullier reviews previous work on and describes lateral organs in the Orbiniidae, Spionidae, Scalibregmidae, Opheliidae, Capitellidae, Trochochaetidae, Flabelligeridae, and Ampharetidae.

In the course of making observations on living material with the higher magnifications of a stereoscopic dissecting microscope, it was noted that in the region between dorsal and ventral setal fascicles, both in the anterior and the posterior regions, with the exception of the ninth setiger, there appear to be fascicles of very fine capillary setae projecting at right angles to the longitudinal axis of the body. Later observations of preserved intact specimens and sectioned material failed to demonstrate the presence of setal fascicles in these areas; all that could be seen was the so-called "lateral organ" of Jones (1963). More critical observations of intact living animals did, in fact, confirm that what had been assumed to be capillary setae were, indeed, long, non-motile stereocilia projecting from the lateral organ (Figs. 3, 4, and 6, LO). In healthy specimens these stereocilia extend straight out, but in the case of morbid or dying animals, the stereocilia are seen to undergo apparently random waving movements, approximately one complete wave movement in two seconds. In sectioned material the stereocilia lose their rigidity (Figs. 28 and 29).

Histological examinations of the lateral organs reveal somewhat more detail than was possible earlier (Jones, 1963). Full-face views of the lateral organ (Fig. 27) show an ordered network of fine granules arranged in approximately 18 parallel rows separated in the midline by a single row of larger granules; these are in the transverse plane of the worm. The granules, which appear as dots, may coincide with the point of emergence of the extended stereocilia, or may, indeed, be the basal granules of the stereocilia. Cross-sections of the lateral organs (Fig. 29), in the frontal plane of the whole animal, show that this structure is actually made up of an anterior and posterior half, and that fibers (ciliary rootlets?) running internally are separated by a row of large, relatively clear nuclei which occur just under the longitudinal axis of the lateral organ. Internal to these nuclei the fibers merge into what may be a sub-epidermal nerve plexus and they can not be traced further. No obvious nerves were seen in association with the lateral organs. In longitudinal sections of the lateral organs (Fig. 29), the same view is seen as noted previously (Jones, 1963, Fig. 70). The cuticle becomes thinner as it covers the lateral organ and is underlain by at least three layers. The outermost is clear and structureless, except for fibers which seem to connect the cilia with the deeper parts of the organ. The second layer bears rod-like structures which appear to be swellings of the fibers. The third layer is deeply stained with the acid fuchsin of Mallory's triple stain and appears to be composed of fibrous material oriented parallel to the outer surface. There are faint indications of other deeper layers, but these were not resolvable. The main mass of the internal portion of the lateral organ is composed of numerous fibers oriented perpendicular to the outer surface. As mentioned above, these appear to be internal extensions of the external cilia. Two kinds of nuclei are present approximately half way along the length of these fibers. The first is the large, clear type seen in cross-sections of the organ (Fig. 29), and the second is somewhat smaller and more darkly staining. There is a third type of structure to be seen at the internal ends of the fibers, but the fine details of these could not be made out. They have an elongate teardrop-shape, appear to be uniformly and finely granular, and stain greenish-brown in Mallory's triple. Their outer, attenuated ends appear to give rise to some of the fibers which comprise the central area of the lateral organ. While these granular structures were seen in longitudinal sections of the lateral organ (Fig. 28), they were not noted in cross-sections (Fig. 29).

It has been noted previously (Jones, 1963, p. 28) that the lateral organs of the magelonids bear a strong resemblance to those of certain orbiniids and spionids, as described by Rullier (1951). The three layers of the cuticle described by Rullier (1951, p. 228, Fig. 6A) for the lateral organs of the Orbiniidae are present in those of the Magelonidae. The larger clear nuclei associated with the internal fibers are probably comparable to Rullier's "grandes cellules sensorielles." The granular structures mentioned above may well prove to be glandular cells, similar to those described by Rullier (1951, pp. 224–225, Fig. 5C) for the dorsal organs of *Orbinia* (*Phylo*) *foetida* (= *Aricia foetida*). It is interesting to note that cross-sections of the lateral organ of *Magelona* sp. are quite reminiscent of the ciliated cells of the dorsal organ of *Orbinia latreilli* (= *Aricia latreillii*) and the cells of the dorsal ciliated bands of the spionid, *Malacoceros fuliginosus* (= *Scolelepis fuliginosa*). In both of the latter cases, individual ciliated cells contain a single set of internal fibers which narrows as it approaches the nucleus. The cross-sections of the lateral organs of *Magelona* sp. (Fig. 29), while showing no cell membrane surrounding the anterior and posterior parts, exhibit the same type of basal restriction of the two masses of fibers in the vicinity of the large clear nuclei. Of the various ciliated sensory structures observed by Rullier, the lateral organs of *Magelona* sp. appear to be most closely related to the lateral organs of the Orbiniidae, in general, and of *Scoloplos armiger,* in particular.

It has been noted that intact specimens of *Magelona* sp., when living in their burrows, are quite sensitive to shocks and sudden vibrations. It has been suggested by Horridge (1966) that certain structures, found in ctenophores and chaetognaths, are vibration receptors. These structures are similar in several ways to the lateral organs of *Magelona* sp. and it may well be that the lateral organs are the receptors which initiate the sudden response to vibrations in the substrate. Their position, one pair located laterally on each segment, with their extended non-motile cilia in close proximity to or in contact with the sides of their burrow, is well-situated for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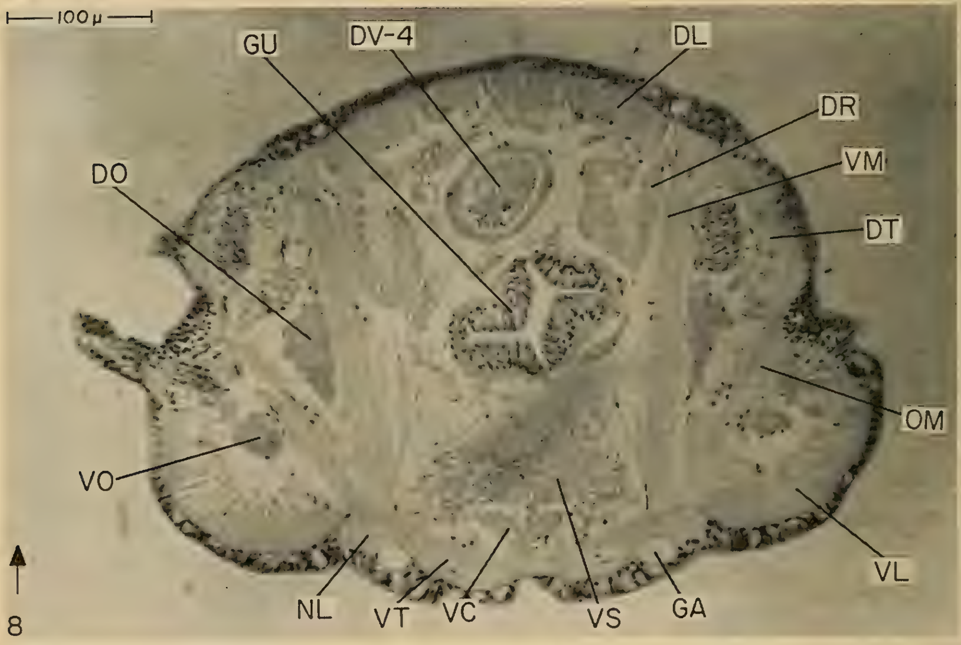
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FIGURE 7. Frontal section of setigerous segments 5 to 10; haematoxylin and eosin; arrow should point to the right.

FIGURE 8. Transverse section at the level of the fifth setiger; haematoxylin and eosin.

this function. In addition, the so-called palpocils noted by McIntosh on the tips of the tentacular papillae (see below) may well represent some other kind of sensory receptor, perhaps for the reception of vibration stimuli through the overlying water.

NERVOUS SYSTEM

The supraoesophageal ganglion (Fig. 24, SG) is situated in the basal region of the prostomium. Connectives (extensions of the "vorderes Schlundkonnektiv" of Orrhage, 1966), extend laterally and posteriorly, just internal to the epithelial cells underlying the cuticle. They are lateroventral at the level of the first setiger and remain so (Figs. 8 and 9, NL) until the seventh setiger where they begin to approach one another. Associated giant axons (Figs. 8 and 9, GA) fuse medially by the eighth setiger and the neuropiles of the ventral nerve cords fuse in the ninth setiger; these fusions persist throughout the posterior region (Fig. 10, NL and GA). There appear to be no discrete segmental ganglia associated with the ventral nerve cord and cell bodies are scattered along its length. In the anterior region no transverse commissures were seen connecting the separated nerve cords.

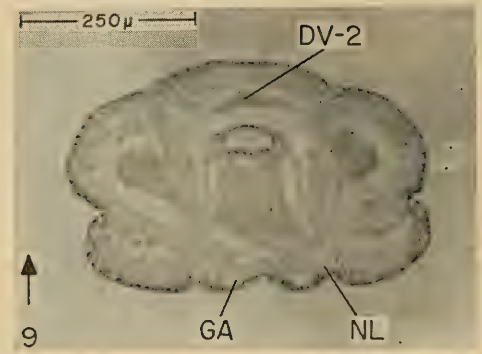
It should be noted that there are indications that the supraoesophageal ganglion is not well-demarcated or clearly confined to a specific area. On the contrary, it appears that this ganglionic mass merges with and gives rise to a nerve plexus which runs below the epithelial layer of the prostomium-peristomium and may continue into the rest of the anterior part of the body, if not the whole length of the animal.

MUSCULAR SYSTEM AND SEPTA

All of the somatic muscles of *Magelona* sp. appear to be striated and/or cross-striated, as noted by Romieu (1923), and, further, appear to be of the "nematode-type," as described by Orrhage (1962), in that muscle nuclei are removed from the muscle fibers and invested by a peripheral sarcoplasm; the complexity of disposition of the muscle bundles of the anterior region foreshadows the complexity of the circulatory system.

The prostomium is provided with two pairs of elongate structures (Fig. 1, PM) which terminate sub-marginally at the anterior end of the prostomium (Fig. 1, PS). These have been referred to as ridges, muscle bands, "crêtes longitudinales," and "rebordes redondeados." In reality these are hollow, presumably fluid-filled, cylindroids whose inner surfaces, particularly dorsally and ventrally, are provided with longitudinal muscles. The medial pair of muscular cylinders ends in the vicinity of the supraoesophageal ganglion. The lateral pair continues posteriorly past the prostomial-peristomial region and emerges in the coelomic cavity of the anterior region where their associated muscles are confluent with the ventral longitudinal muscles (Fig. 8, VL). The function of the muscular cylindroids would seem to be that of providing rigidity to the prostomium, particularly during burrowing activities. The hydrostatic pressure of the coelomic fluid could be readily transferred to these prostomial structures and the turgor thus established would serve to render the prostomium relatively inflexible and more efficient for burrowing.

Ventral transverse muscles are found just anterior and just posterior to the mouth, while dorsal transverse muscles are located posterior to the mouth at about the level of the origin of the tentacles (Figs. 1 and 2, TE). In the anterior region, paired, segmentally arranged vertical muscles (Figs. 7 and 8, VM) extend from the internal surface of the dorsal body wall to the dorsal surface of the nerve cords, and oblique muscles (Figs. 8 and 9, OM) extend from the nerve cords to the internal surface of the mid-lateral body wall. The vertical muscles are restricted to single bundles of muscle fibers extending from just anterior to a given setal fascicle to about three-quarters of the intersegmental length to the next posterior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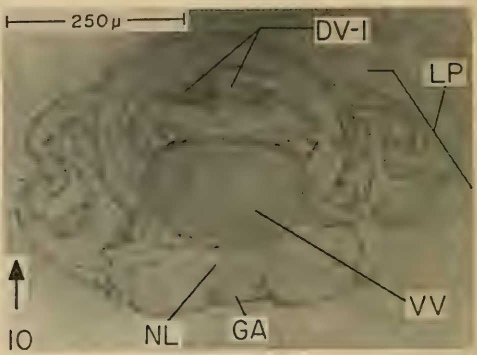
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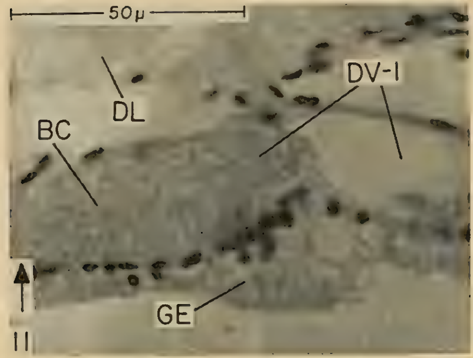
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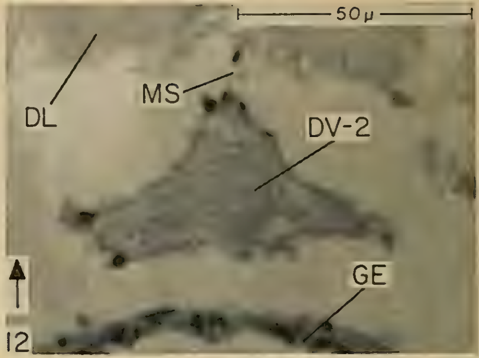
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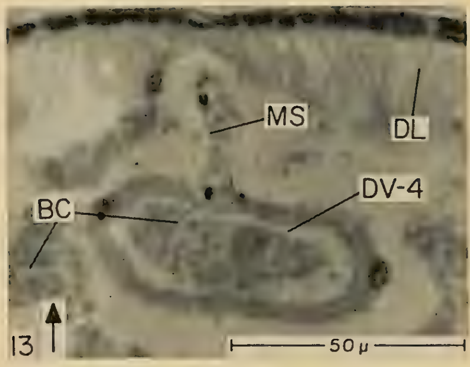
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DV-4
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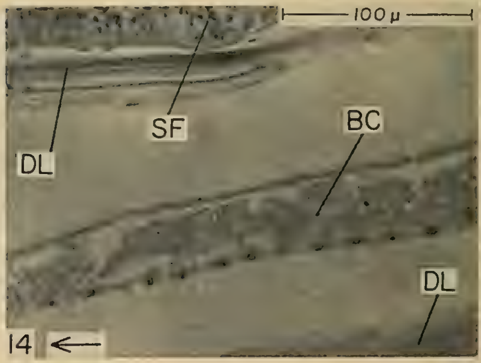
100μ
SF
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14

FIGURE 9. Transverse section between setigers 7 and 8; haematoxylin and eosin.

FIGURE 10. Transverse section between setigers 10 and 11; haematoxylin and eosin.

FIGURE 11. Transverse section of dorsal vessels of posterior region (DV-1) showing differential filling of the vessels due to action of valves of posterior region; haematoxylin and eosin.

FIGURE 12. Transverse section of dorsal vessel between setigers 8 and 9 (DV-2); haematoxylin and eosin.

FIGURE 13. Transverse section of dorsal vessel at the level of the fifth setiger (DV-4); haematoxylin and eosin.

FIGURE 14. Frontal section of dorsal vessel (DV-4) in the vicinity of the third setiger; haematoxylin and eosin.

setal fascicle. The oblique muscles occur in smaller bundles, about ten per segment, which generally follow the disposition of the vertical muscles. The dorsolateral body walls, between the oblique and vertical muscles, are provided with transverse muscles (Figs. 8 and 9, DT); similar transverse muscles extend between the ventral attachment points of the vertical muscles (Figs. 8 and 9, VT). Dorsal and ventral longitudinal muscles (Figs. 8, 9, and 10, DL and VL) are mid-dorsal and ventrolateral, respectively. There are other muscles, the larger dorsal proboscidial retractors (Figs. 7 and 8, DR) and the ventral medial longitudinal muscles (Figs. 8 and 9, VC). The dorsal retractors originate at about the level of the sixth setiger from the inner surface of the dorsal longitudinal muscles, extend anteriorly and ventrally, and insert on the inner surface of the extruded proboscis at its most anterior point. The ventral medial longitudinal muscles originate from the ventral surface of the ventral blood sinus as a flattened muscle layer between the eighth and ninth setigers and extend anteriorly as separate muscle bundles, wing-shaped in cross-section. As these longitudinal muscles approach the proboscis they become flattened once more and pass over the ventral transverse muscle posterior to the mouth. An anterior extension of the ventral medial longitudinal muscles extends into the extruded proboscis and inserts on its ventral, basal, inner surface and acts as a ventral retractor. Throughout their length, the ventral medial longitudinal muscles are confined within the ventral blood sinus which is confluent with the cavity of the extruded proboscis. The dorsal retractors are found in individual sinuses which are dorsoposterior extensions of the ventral sinus, and which separate from the latter at about the level of the sixth setiger. Anterior to this point the dorsal retractors also lie in the ventral sinus.

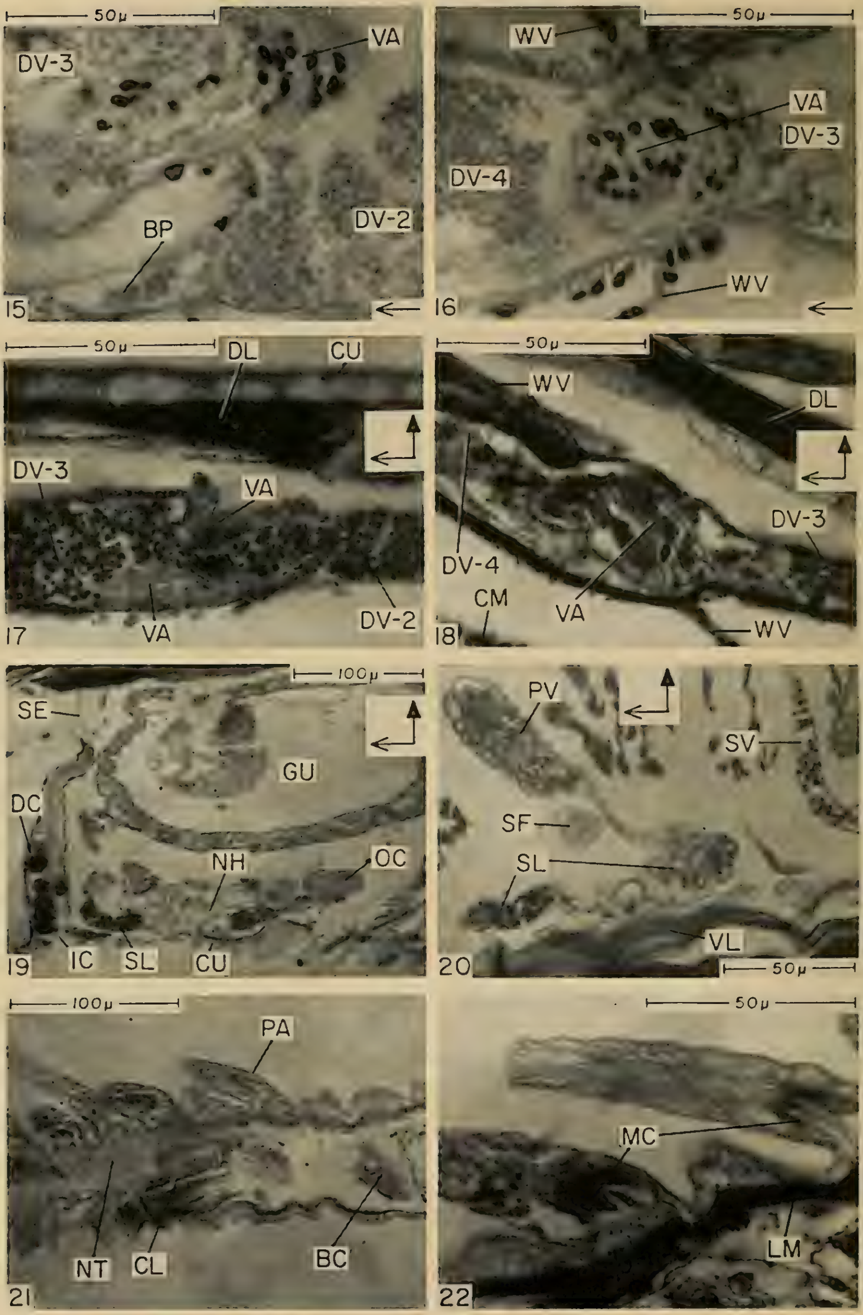
Throughout the length of the anterior region there are no septa. The ninth setiger, however, is provided with complete septa which form the base for two pairs of vertical muscle sheets; these line the anterior and posterior internal walls of the segment; there is another set of vertical muscles immediately adjacent to the gut (Fig. 25). Complete septa set off all segments of the posterior region. These are not provided with muscle sheets and appear to consist of two thin layers of coelomic epithelium.

In the region of the ninth setiger the dorsal and ventral longitudinal muscles become enlarged while the oblique and the lateral transverse muscles are diminished and the vertical and ventral transverse muscles disappear. In the posterior region the longitudinal muscles are further enlarged and the oblique and lateral transverse muscles are further diminished (Fig. 10).

The musculature of the anterior region of the body gives a strong PAS reaction which does not disappear after diastase digestion. In contrast, with the exception of the setal muscles, the musculature of the posterior region does not give a PAS/AB reaction.

It should be noted that all muscles which have an origin or insertion on the body wall are attached to a sheet of connective tissue underlying the dermis (PAS-positive after diastase digestion).

It would seem that McIntosh was not aware which were the proboscidial retractor muscles, for it was the ventral medial longitudinal muscles which he labeled as such (McIntosh, 1878, Pl. 32, Fig. 4) and the true retractors were labeled as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DV-3
VA
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DV-2
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PV
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100μ
PA
NT
CL
BC
21
50μ
MC
LM
22

Figures 15–22.

muscles inserting on the gut. In addition, McIntosh felt that the ventral medial longitudinal muscles were in direct connection with the ventral transverse muscle posterior to the mouth. It has been noted that these longitudinal muscles remain separate from the ventral transverse muscle and, as the ventral retractors, insert on the internal, ventral, basal surface of the extruded proboscis; the function of these ventral retractors would seem to be that of initiating the inward movement of the returning proboscis.

Although it is probably merely a semantic point, it might be noted that McIntosh seemed to feel that the basic muscular structure of the anterior region is a fan-shaped triangular mass of muscle fibers which radiate dorsolaterally from the vicinity of the laterally placed ventral nerve cords. McIntosh (1877, p. 436) goes on to state that ". . . the fan-shaped mass of fibres is split into two by the intervention of the blood-channel [the "external lateral trunk"]" and that the ". . . interposition of the blood channel in the great fan-shaped anterior muscle . . . separates it into a vertical and oblique division." The implication here is that the lateral out-pocketings of the ventral blood sinus are instrumental in the formation of the vertical and oblique muscle series. It would seem, however, that these two series are formed as such and that it is only in the extreme anterior region that they are even in close proximity to one another.

CIRCULATORY SYSTEM

The blood (Figs. 11, 12, *et seq.*, BC), which contains enucleate corpuscles 1–2 μ in diameter, returns anteriorly from the posterior region and from segmental vessels, passing through a pair of dorsal vessels in the posterior region (DV-1). These are relatively thin-walled (Figs. 10 and 11, DV-1), closely applied to one another and to the dorsal surface of the gut, and supported by a mid-dorsal mesentery. Blood flow appears to be maintained by non-peristaltic, anteriorly directed, contractions of the muscles of the gut wall, apparently in much the same manner as noted by Hanson (1950, p. 114) among the serpulids. In some cases it has been noted that the medial separation between the two dorsal vessels is not complete, for there may be a flow of blood from one to the other. In the region of the ninth to tenth setigers, the medial wall between the two dorsal vessels disappears and the resulting single dorsal vessel narrows as it passes anteriorly. Between the seventh and ninth setigers the dorsal vessel (Figs. 9 and 12, DV-2) is thin-walled and enlarged; at the level of the seventh setiger it gives rise to a pair of anteriorly

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FIGURE 15. Frontal section of the valve separating DV-2 and DV-3; haematoxylin and eosin.

FIGURE 16. Frontal section of the valve separating DV-3 and DV-4; haematoxylin and eosin.

FIGURE 17. Sagittal section of the valve separating DV-2 and DV-3; Mallory's triple.

FIGURE 18. Sagittal section of the valve separating DV-3 and DV-4; Mallory's triple.

FIGURE 19. Sagittal section of the ventrolateral area of a posterior segment showing the relationship of the segmental loop, oöcytes, and the presumed nephridium; Mallory's triple.

FIGURE 20. Sagittal section of a posterior segment showing the relationship of the pulsatile vesicle to the posterior portion of the segmental loop; Mallory's triple.

FIGURE 21. Oblique longitudinal section of the distal portion of a prostomial tentacle; Mallory's triple.

FIGURE 22. Longitudinal section of tentacular papillae; Malloy's triple.

directed branches (Figs. 7 and 15, BP), and then narrows. Anterior to this constriction the dorsal vessel once more enlarges (Fig. 7, DV-3), has a somewhat thicker wall, and, just posterior to the sixth setiger enters the dorsal portion of the ventral sinus whose walls (Figs. 7, 16, and 18, WV) are firmly attached to the dorsal vessel; at this point there is yet another constriction. Anterior to the latter the dorsal vessel is thick-walled (Figs. 7, 8, 13, and 14, DV-4), and maintains a uniform diameter. As the dorsal vessel (DV-4), approaches the level of the tentacles, it bifurcates and the two branches pass laterally, as afferent vessels (Fig. 24, AT), through the tentacular bases, and along the length of the tentacles to their tips, where the vessels loop back upon themselves and, as efferent tentacular vessels, return to the body. There are numerous cross-connecting capillaries between the afferent and efferent tentacular vessels in the distal portion of the tentacles. The efferent tentacular vessels open into an anteriorly directed extension of the ventral sinus which terminates in a ring-like vessel (Fig. 24, RV) surrounding the supraoesophageal ganglion. Anteromedially in the ring is a partition which restricts movement of blood around the supraoesophageal ganglion. Blood returning to the body from the tentacles may move posteriorly through a connection to the ventral sinus, provided with up to five short lateral branches, and then into the ventral sinus, or it may pass anteriorly to the region of the supraoesophageal ganglion.

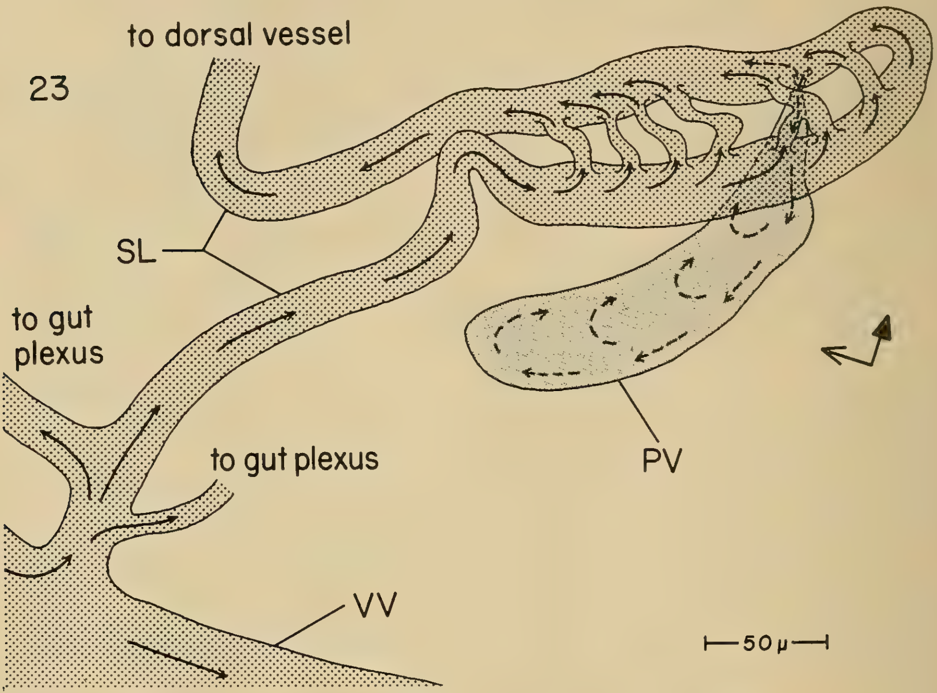

Figure 23. Diagrammatic view of a segmental loop and its associated pulsatile vesicle showing the flow of blood; drawn from life.

Blood passes into the ventral sinus and may then move into any of the lateral outpocketings (Fig. 24, DO), passing between the segmentally-arranged vertical muscle bundles, or may pass posteriorly to the ventral vessel of the posterior region or, by way of the pair of dorsal bypass branches, back to the dorsal vessel. Anteriorly, the ventral sinus surrounds the gut and contains the two pairs of proboscidial retractor muscles. Posteriorly, at about the level of the fifth or sixth setiger, the dorsal retractors leave the blood sinus and the sinus itself is restricted to the ventral surface of the gut. At about the level of the eighth or ninth setigers, the ventral sinus becomes separated from the gut and gives rise to a pair of dorsal blind diverticula which lie between it and the gut; the ventral sinus has now become a distinct ventral vessel. The ventral vessel may undergo a series of alternating enlargements and constrictions, roughly related to segmental boundaries, and, at the level of the fifteenth setiger it becomes quite restricted; this restriction of cross-sectional area is maintained throughout the rest of the body.

In the anterior region, as the ventral sinus passes the spaces between vertical muscle bundles, lateral outpocketings pass from the sinus in the central body cavity, to the area demarcated by the vertical and the oblique muscles (Figs. 7, 8, and 9, DO). These outpocketings are segmentally arranged and appear to be quite extensible pouches; they, in turn, give rise, ventrally, to similar outpocketings to the area ventral and lateral to the oblique muscles (Figs. 8 and 9, VO). The lateral pouches and the sinus at the level of the sixth setiger also open to the sinus extensions surrounding the dorsal retractors and receive the pair of branches arising from the dorsal vessel.

In the anterior ends of nearly all segments of the posterior region, the ventral vessel gives rise to paired segmental vessels which, immediately after leaving the ventral vessel, further give rise to branches which go directly to the gut and ramify over its surface to form a plexus. The main segmental vessel proceeds laterally in close association with the adjacent septal wall. At approximately half the distance to the lateral body wall, the segmental vessel leaves the septum, turns posteriorly for a short distance and doubles back on itself (Fig. 25, SL). From six to eight cross-connections form a kind of capillary bed across the loop so formed (Fig. 23). In the posterior third of the loop, a large extensible vesicle is attached to the returning portion of the vessel (Fig. 20 and 23, PV). This is the contractile vesicle noted by Müller (1858, p. 215) and McIntosh (1877, p. 150; 1879, pp. 341–342; and 1915, pp. 221–222). The efferent segmental vessel proceeds anteriorly to the septum and appears to lie between the two layers of the septum as it moves dorsally and then medially to join with the dorsal vessel (Fig. 20, SV). It should be noted that gonadal tissues (Fig. 19, OC) are located just ventral to the loop and capillary bed, and that, in the same region, there is a mass of tissue whose function is unknown, but whose appearance suggests that it may prove to be the nephridium (Fig. 19, NH). It is of interest to note that although the wall of the vesicle appears to be acellular and stains blue with aniline blue, there appears to be an intrinsic contractility associated with this structure; it may be that the characteristic scalloped appearance of its wall is due to the presence of fine circular muscle fibers which have not, as yet, been demonstrated (Fig. 20, PV). Although the vesicle usually fills with blood before it contracts, on several occasions it did not, but underwent contractions even though unfilled with blood. The vesicle was

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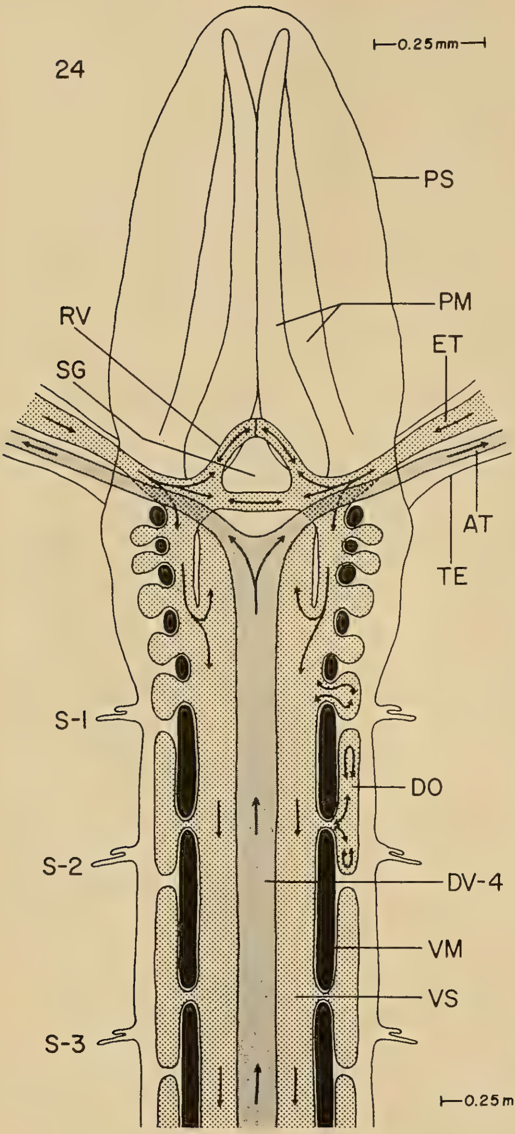
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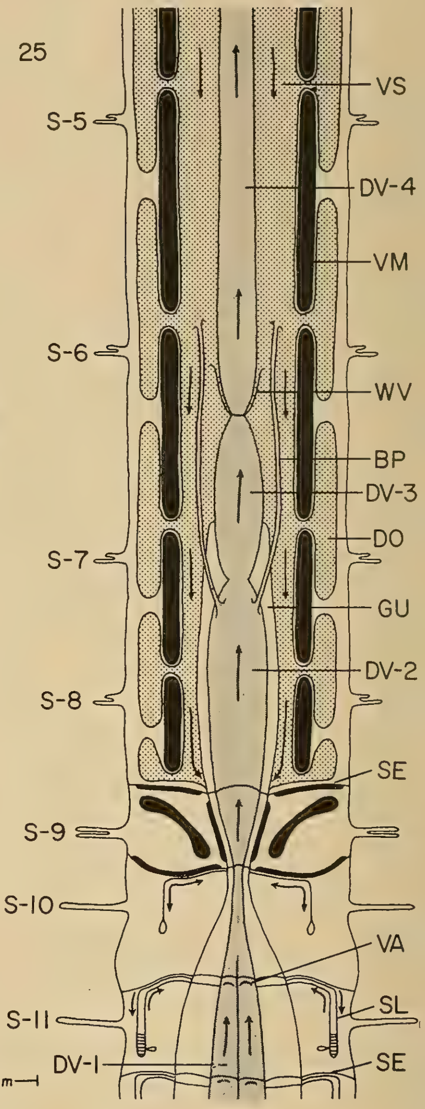
VS
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S-11
DV-1
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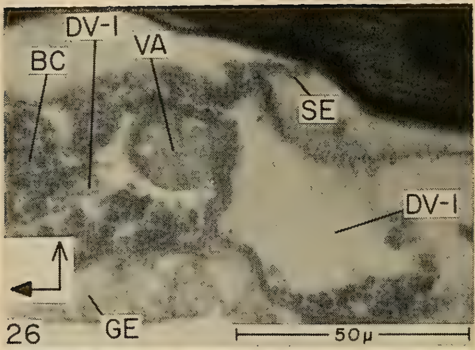
FIGURE 24. Diagrammatic dorsal view of prostimum, peristomium, and anterior three setigerous segments of *Magelona* sp. showing circulatory elements (stippled) and the vertical muscles (solid); arrows indicate net blood movement; blood moving forward from the ventral sinus (VS) in the area ventral to the bifurcation of the dorsal vessel may move into the base of the proboscis for its extrusion.

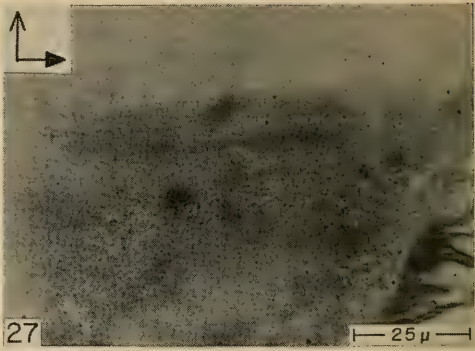
FIGURE 25. Diagrammatic dorsal view of setigerous segments 5 to 11; arrows, stippling and solid areas as in Figure 24.

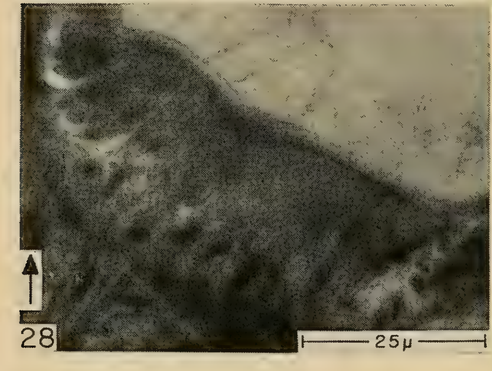
seen to pulsate fom 23 to 26 times per minute. The tenth segment is not provided with a full loop system for there are only segmental vessels from the ventral vessel which end blindly in a pulsating vesicle (Fig. 25).

When living *Magelona* sp. are observed, it is possible to note the functioning of the circulatory system and its interaction with the musculature, particularly in the anterior region of the body. When the proboscis of a given animal is not everted, there is a continual ebbing and flowing of blood between the ventral sinus and its lateral outpocketings in the anterior region. The net movement of blood in this case is posterior in the ventral sinus. It has been noted above that the cavity of the everted proboscis is in free communication with the ventral sinus and its segmental, lateral outpocketings; thus, the proboscis is inflated primarily by the hydrostatic pressure of the blood, not the coelomic fluid, as is the case in most burrowing polychaetes. The coelomic fluid also functions, to a limited extent, in the protrusion of the proboscis, for sectioned material shows that the dorsal medial region of the extruded proboscis (Fig. 31, CC) is an extension of the coelomic cavity which lies between the gut and the ventral sinus. The restriction of blood within the proboscis has been noted previously by McIntosh (1878, Pl. 37, Fig. 1) and Dales (1962, Fig. 16C), but neither noted the presence of an extension of the coelomic cavity. The protrusion of the proboscis appears to be brought about by a general contraction of all muscles of the anterior region, except the retractors, and, with the extrusion of the proboscis, the anterior region changes from an opaque pink to nearly transparent as the blood is forced forward. Under these conditions it can readily be observed that, not only does blood still move from the posterior region, but that it is passed forward rhythmically. Further, there is an interrelated alternation of swelling and contraction of the posterior enlargement of the dorsal vessel (DV-2) and the anterior enlargement (DV-3) and a correlated total contraction of the anterior portion of the dorsal vessel (DV-4).

Histological observations have demonstrated that the closing off of DV-3 is accomplished by valves between DV-3 and DV-2 (Figs. 15 and 17, VA) and between DV-3 and DV-4 (Figs. 16 and 18, VA). The valves appear to be composed of numerous projections of connective tissue (staining with alcian blue and aniline blue) which extend into the vascular lumen from the ventral and lateral walls of the vessel; there is a dorsal cushion of the same material against which the projections impinge. No discrete valves or valve-like structures were noted between DV-2 and DV-1 in the ninth segment; apparently the restricted cross-sectional area and muscles of the vessel wall are adequate to form a closure, and opening is probably accomplished by contractions of the muscles of the ventral vessel and its dorsal diverticula, which are closely associated with the dorsal vessel at this point. Histological cross-sections of DV-2 (Fig. 9) show that there is very little wall musculature which might account for forward movement of the blood. DV-3, on the other hand, is provided with both longitudinal and circular muscles and these would seem to provide sufficient hydrostatic pressure to move the blood into DV-4. Movement of the blood from DV-2 to DV-3 is probably accomplished by pressure from the blood in the posterior body region as the posterior valve opens or by the elasticity of the enlarging DV-3 as the anterior valve closes. DV-2 may receive blood from the ventral sinus through its pair of branches, as well as from DV-1.







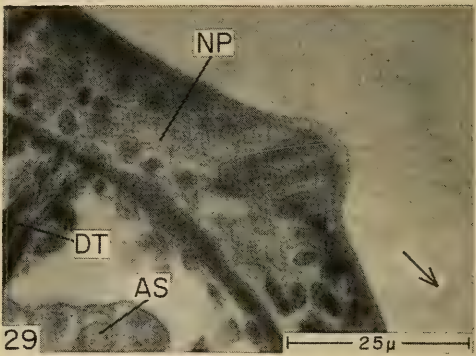


FIGURE 26. Sagittal section of a valve in one of the posterior dorsal vessels (DV-1) showing the action of the valve in preventing backflow of the blood; Mallory's triple.

FIGURE 27. Section showing a full-face view of the surface of a lateral organ of setiger 10 (= sagittal section of the whole animal); Mallory's triple.

FIGURE 28. Longitudinal section of a lateral organ of setiger 7 (= transverse section of whole animal); Mallory's triple.

FIGURE 29. Cross-section of a lateral organ of setiger 10 (= frontal section of whole animal); Mallory's triple.

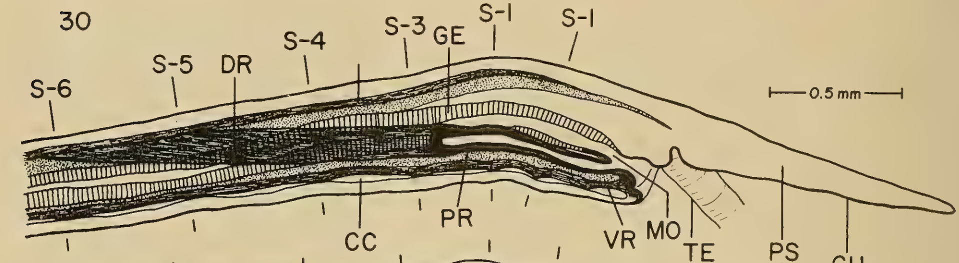
The vessel wall of DV-4 (Fig. 13) is greatly thickened (from 4 to 7 μ) and three layers can be observed, *i.e.,* an internal, apparently elastic layer, a layer of muscle fibers, and an external layer of sarcoplasm in which the muscle nuclei are situated. The structure of this part of the dorsal vessel is of further interest in that longitudinal sections reveal that the muscle fibers are restricted in an anterior-posterior direction (in approximately 15 to 20 μ subsections) and that they appear to be independent of adjacent groups of fibers (Fig. 14). In addition, properly oriented cross-sections show two, and sometimes three, nuclei in the same plane, and longitudinal sections show that nuclei are restricted in the same manner as the muscle fibers (Fig. 14). It would seem, then, that this section of the dorsal vessel (DV-4), in essence, is comprised of an elastic tube with either binucleate, circular muscle cells or paired, uninucleate, "C"-shaped muscle cells arranged along its length.

While attempting to trace the course of blood from the segmental loops to the dorsal vessel of living animals, it was noted that there appeared to be a rhythmic ebb and flow of blood in the posterior dorsal vessel (DV-1). Sagittal sections through the dorsal vessel reveal that there are segmental valves in each of the dorsal vessels at or just anterior to each septum (Fig. 26). These valves differ from those of the anterior dorsal vessel in that they appear to operate as "flapper valves," in contrast to the sphincter-type of the anterior dorsal vessel, and prevent a back flow of blood merely by pivoting about a ventral basal attachment. It was noted that the segmental vessels from the loops empty into the dorsal vessels just posterior to the "flapper valves" and it is possible that blood from the segmental loops enters the dorsal vessels while the valve is closed and there is little anterior pressure to be overcome by the pulsatile vesicle of the loop system.

Certain observations of the rates of pulsation in various parts of the anterior circulatory system have been made. It appears that the normal rate in DV-2, DV-3, and DV-4 ranges from 24 to 30 per minute; in the case of the doubled vessels of DV-1, the rate in each is about 14 per minute; the rate of pulsation of the vesicle of the segmental loop is from 19 to 21 per minute, regardless of whether or not the vesicle is filled with blood. It was also noted that when the observed specimen was heated by the microscope illuminator, the pulsation rate in DV-3 rose to about 55 per minute, and when specimens were anaesthesized with isotonic magnesium chloride (1:4 with sea water) the pulsation rate remained unchanged at 24 per minute. Finally, it was noted that when both tentacles were amputated close to their bases, blood flow from DV-3 to DV-4 ceased and blood was shunted from DV-2 directly to the ventral sinus through the paired bypass. After 30 minutes, the cut ends of the tentacles had closed and blood was once more sent from DV-3 through DV-4 to the tentacle stumps, to return to the body by way of the proximal portions of the original efferent tentacular vessels.

The greatest discrepancy in McIntosh's observations on *Magelona* would seem to be that concerned with what he called the "lateral trunks," both internal and external (McIntosh, 1879, pp. 339–340). McIntosh considered that these were open vessels which communicated posteriorly with the ventral vessel; indeed, he seemed puzzled by his observations that ". . . it is difficult to say in what direction the [blood] current in these vessels proceeds . . . [it] does not go in a continuous straight stream backward" (p. 339). This is readily explained by the present observations that McIntosh's external lateral trunks are, in reality, the serially arranged out-pockets from the ventral blood sinus which are located between the vertical and the oblique muscles in each of the segments of the anterior region. Because of their segmental nature, it is clear that there can be no through passage of blood from anterior to posterior. McIntosh's internal lateral trunks would seem to be the lateral portions of the ventral sinus seen below and to the side of the dorsal vessel; here, there is an uninterrupted passage to the ventral vessel but, in living specimens, it has been noted that there is much eddying of the blood as it passes the out-pocketings, and there is fairly obvious net posterior movement of the blood.

The most puzzling of McIntosh's observations is concerned with the location of the pulsatile area of the dorsal vessel. He notes (McIntosh, 1879, pp. 337–339) that the dorsal vessels, at the level of about the eleventh setiger, have ". . . a fine structureless lining membrane, and a conspicuously barred series of striated, circular, muscular fasciculi externally, so that it somewhat resembles a trachea with coiled fibers." As the dorsal vessels approach the tenth setiger, the circular muscles increase in strength and ". . . thus, the great muscularity of these dilatable chambers enables them to perform a heart-like function." In contrast, Mésnil (1896, p. 261) noted that the blood is driven forward by a true heart (un véritable coeur) in the region of the fifth or sixth setiger. Mésnil's account is somewhat confusing, and it may be that he was describing the condition in larval forms, rather than in adults. However, it remains that observations of living specimens of *Magelona* sp. show that the main pulsatile region of the dorsal vessel is the anterior portion (DV-4), while the more posterior chambers (DV-3 and DV-2) appear to be reservoirs which control the volume of blood passing into DV-4 and which also function when the tentacular portion of the circulatory system has been damaged. McIntosh's description, particularly where he mentions the similarity in appearance to tracheae, causes one to wonder whether he might, in fact, have been observing the anterior region of the dorsal vessel. However, it does not seem too likely that he might have made a mistake of five setigerous segments in trying to localize this area of pulsation. Future work on the British *Magelona* will determine if this represents a specific difference between the animals observed or whether it is a *lapsus* on McIntosh's part.



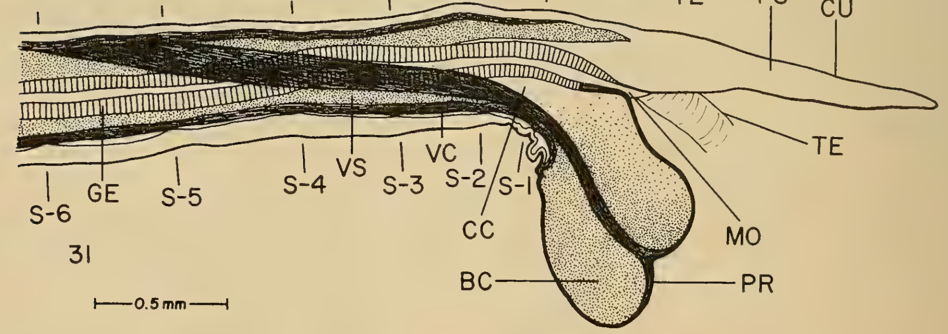


FIGURE 30. Semi-diagrammatic view of a sagittal section of *Magelona* sp., proboscis retracted; unlabelled line between S-3 and S-4 should read "DL" and numbering of setigers should be sequential.

FIGURE 31. Semi-diagrammatic view of a sagittal section of *Magelona* sp., proboscis everted.

In his discussion of the structure and action of the segmental loop system of the posterior region, McIntosh (1879, pp. 341–342) describes the pulsatile vesicle as "suddenly" appearing, as if it ballooned from the loop in some kind of rupturing action; present observations indicate that the vesicle is clearly visible even when the vesicle is not filled with blood and that it does not "suddenly" appear. When the pulsatile vesicle of the segmental loop system was first observed in the present study, it was thought that the contractions of the vesicle might be a response to stretching, due to the distension of the blood-filled sac; however, this would not seem to be the case, for it was noted that even when the vesicle was not filled with blood, it still underwent its usual contractions. This would suggest that the vesicle might be under nervous control, and further, that its rhythmicity might be under the control of some sort of pacemaker. Present histologic preparations have not demonstrated the presence of nervous connections or muscle fibers, although, as mentioned above, the latter may be present and cause the margin of the vesicle to appear scalloped. If muscles ultimately prove to be present, then there exists the possibility of their being myogenic and responsible for the initiation of contractions.

It would seem that the structure of the dorsal vessel of *Magelona* sp. is rather unique, insofar as its tubular type of muscle cell is concerned; however, such "drain

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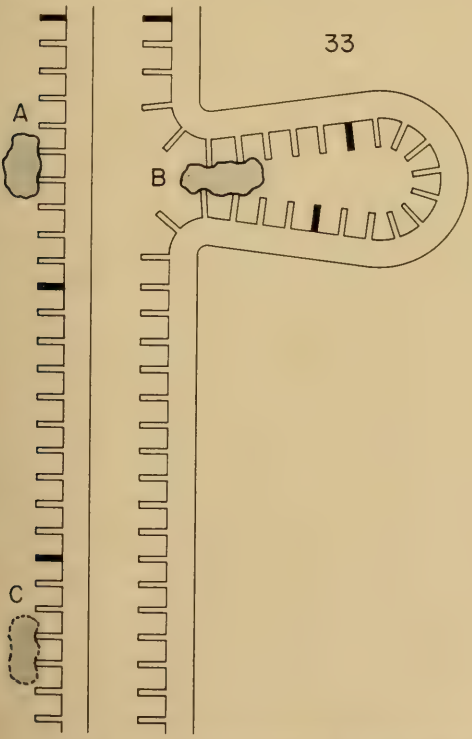

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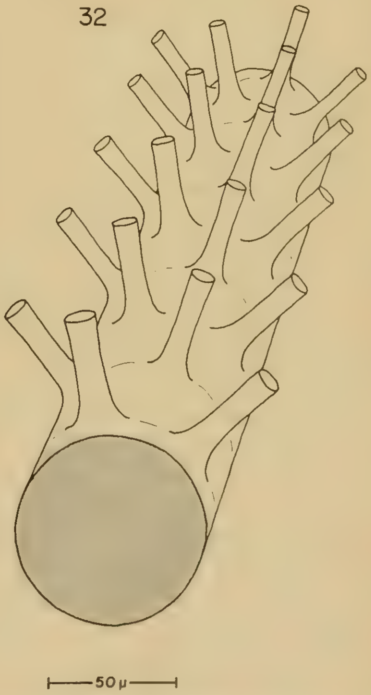

FIGURE 32. Diagram showing the arrangement of papillae along the length of a prostomial tentacle.

FIGURE 33. Diagram showing the movement of food material along the length of the papillated portion of a prostomial tentacle; the same papillae in each diagram are solid.

pipe" or "ring" cells have been noted in the dorsal branchial vessels of the polychaete *Sternaspis* by Goodrich (1905) and in the dorsal vessels of the naidid oligochaetes *Chaetogaster diaphanus* and *Stylaria proboscidea,* as well as in an unidentified tubificid, by Bergh (1900). In addition, the serial arrangement of muscle cell nuclei, which appears to be associated with this type of vascular cell, has been noted in the above species, as well as in the following polychaetes: *Lepidonotus, Nephtys, Glycera,* and *Arenicola* (by Retzius, 1891), *Nereis* (by Retzius, 1905), and in serpulids (by Hanson, 1949, 1950, and 1951).

Burrowing, Feeding and Behavior

When freshly collected specimens are allowed to burrow in an observation chamber, consisting of a thin layer of sand between two pieces of glass, it is possible to observe the burrowing and feeding activities of *Magelona* sp. In the case of the former, it is seen that the worms insert their thin, flat prostomium into the substrate with a sinuous, lateral, as well as a forward, movement. When the head is entirely covered, the proboscis is extruded and acts as an anchor as the remainder of the body is brought forward. The proboscis is retracted and the prostomium is inserted further into the sand, the purchase for this being afforded by the posterior part of the body. This cycle is repeated until the worm has completed its burrow. At the beginning of burrowing, it is noted that the worms almost invariably move straight down until they encounter the bottom of the observation chamber and then upward until they reach the surface of the sand. During the formation of the burrow, the paired tentacles trail back along the sides of the body and do not take part in the burrowing operation. After the burrow is complete, it is noted that the worms are extremely sensitive to vibrational stimuli, both in the substrate and the overlying water.

The integrity of the burrow wall appears to be established and maintained by mucous secretions, most probably formed from dermal inclusions which are distributed dorsolaterally and ventrolaterally throughout the length of the anterior region. In addition, intersegmental areas (Fig. 19, IC) and the tips of the lamellae of the posterior region are provided with concentrations of similar inclusions. When at rest in the burrow, the tips of the lamellae and the dorsal and ventral surfaces of the anterior region are in contact with the burrow wall.

When the worm reaches the surface of the sand, the body is withdrawn several millimeters below the surface and the tentacles, which are not withdrawn, are thrown into loops which enlarge until the tentacles are entirely free of the burrow. The tentacles are then extended to as much as 15 to 20 mm. and wave freely in the overlying water. The papillae, confined to the distal four-fifths of the tentacular length, are fully extended, and it is seen that each papilla is separated from its nearest neighbor by approximately its own length. In the fully-extended condition, it is noted that there are four rows of papillae along the length of the tentacles, and that the outer rows are at approximately right angles to one another (Fig. 32). It is further seen that there is a paired, rather than a quartet arrangement of the papillae in a transverse direction, *i.e.,* outer papillae are lined up with inner papillae of the opposite side, not the nearest inner papillae.

After the worms had been living in the observation chamber for several weeks, it was noted that the inner surfaces of the glass plates had a thin layer of diatoms and detritus which had settled out from the circulating unfiltered sea water. It was further noted that, while the layer of material settled on the glass was fairly uniform, there was an area extending from the sand surface upward about 10 mm. which was entirely free of the fouling material. Since the several specimens of *Magelona* sp. were the only animals in the chamber, it seemed obvious that the cleaned area represented feeding areas of these worms. Although the worms were observed *in situ* with a low power microscope, they were never unequivocally observed to feed on the settled material on the sides of the observation chamber. In an attempt to elicit a feeding response, flocculent material, consisting of miscellaneous organic material settled on the bottom of sea tables from unfiltered sea water, was pipetted into the chambers. There was an almost immediate response on the part of the burrowed worms, in that the extended tentacles were agitatedly waved about and when contact was made with a mass of debris (Fig. 33, A) the tentacle was thrown into a loop just proximal to the point of attachment of the debris (Fig. 33, B). The attached material was placed in contact with the papillae on the opposite side of the loop and the tentacle was straightened (Fig. 33, C). A second loop was then formed proximal to the new point of attachment, and a second transfer was effected to a new set of papillae; the tentacle was again straightened, and further loops were formed until the mass of debris had progressed the length of the papillated portion of the tentacle. When the non-papillated region (the proximal 20% of the tentacular length) was reached, the prospective food material seemed to undergo a free fall directly into the mouth. It was not possible to follow the subsequent passage of this food material in detail, but it was noted that the passage was fairly rapid through the first nine setigers. Similar results were obtained when eggs and nauplii of *Artemia* were introduced into the chamber. When the opaque eggs were ingested, it was possible to observe through the body wall that food material does, indeed, pass quite rapidly through the anterior region. Further, it was noted that the passage was not always smooth, but there appeared to be a series of oscillations, with a net posterior movement. It also was observed that when food material oscillated in the gut, precisely similar oscillations were to be seen in the movement of new food material in its transfer from the end of the papillated area of the tentacles to the mouth.

The papillae (Figs. 21 and 22, PA) are solid structures throughout their length. Their primary musculature consists of longitudinal muscle fibers, although, from their movements in the intact animal, there must be a complement of circular muscles, as well. At the bases of the papillae there are a number of cells which stain negatively with PAS and positively with alcian blue, and which are assumed to be mucus-secreting (Fig. 22, MC), probably an acid mucopolysaccharide. Similarly stained thin passages can be seen leading from these basal cells to the flattened tips of the papillae. The proximal 20% of the tentacles and the ventral surface of the prostomium are also provided with apparently mucus-secreting cells, exhibiting the same staining reaction. On one occasion short, non-motile, cilia-like structures were seen at the tip of the papillae of living *Magelona* sp. Subsequent observations of both living material and sectioned material have failed to confirm this observation, so it is possible that these might be merely extruded mucous threads or some other such artifact.

Final observations concerning the behavior of *Magelona* sp. were made on undisturbed specimens in their burrows. From time to time, it was noted that the anterior 20 to 25 setigers underwent lateral sinuous movements within the burrow, approximately 85–100 per minute. The addition of carmine particles confirmed that an inward-moving current was set up by these undulations. Such movements were seen in rather few individuals at any one time, about one in ten. It was presumed that these movements might have to do with the respiration of the worms, and this assumption seemed warranted in light of the fact that when the supply of fresh overlying water was stopped and the water above allowed to become deoxygenated, nearly all of the specimens in the observation chamber underwent similar lateral pumping motions at an increased rate.

Because of the vascular elements contained in the paired prostomial tentacles, and because of their usual placement in the overlying water, it was further assumed that these structures are active in the respiratory activities of the worms. This was borne out to some extent by the behavior of specimens whose tentacles had been amputated or had been lost during collecting. After having completed a burrow, these animals were frequently seen, extending from their burrow, to the level of the ninth or tenth setiger, undergoing lateral movements, even when there appeared to be an adequate supply of fresh, oxygenated sea water.

In a single preliminary attempt to demonstrate a difference in oxygen uptake, two groups of *Magelona* sp., 60 in each, were observed in a Warburg apparatus at 25° C. The one group whose tentacles had been amputated utilized oxygen at a rate of 1061.5 ml./hr./kg., while the other, the control group, with intact tentacles had a rate of 1036.4 ml./hr./kg.

Several problems are presented by the observations of the feeding of *Magelona* sp. McIntosh's (1911, p. 451) observation that the tips of the papillae bear so-called palpocils which ". . . cover the tip of the papillae like a series of motionless microscopic cilia" agrees with the present single observation of apical cilia on the papillae. However, his suggestion of a sucker-like action of the papillae is somewhat different.

It has been observed that *Magelona* sp. lives in a well-sorted sand environment. This is the result of a relatively high energy habitat with sufficient water movement to create well-defined ripple marks on the surrounding sand surface. The microscopic debris, diatoms, organic debris, and small plants and animals tend to collect at the bottom of these ripple marks and it is felt that this material forms the main source of food for *Magelona* sp. If such is the case, then the insubstantial nature of this material would render a sucker-like action of the papillae rather inefficient in food-getting. The central mass of muscle fibers, which McIntosh suggests would facilitate such a sucker action, might just as well be utilized in the many local fine movements of the papillae which can be observed when the tentacles are extended into the overlying water. The presence of mucous cells associated with the papillae, together with their openings at the tips of the papillae, as well as the movement of newly attached food particles, suggest that the obtaining of food might be through the agency of secreted mucus at the papillar tip. If this is the case, then there must be some means of freeing the food material from the tip of one papilla so that it might be picked up by another when the food is passed down the tentacle. It does not seem likely that the flow of mucus, *per se,* could be stopped quickly or discretely enough to allow this. Three alternative possibilities might be suggested. It is possible that a second secretion might inhibit the flow and/or the adhesiveness of the mucus so that the given food particle would be freed; however, it does not seem probable that such reactions could take place quickly enough to account for the passage of the food particles. The second possibility would require the presence of a sphincter-like band of circular muscles near the tip of each papilla. If contraction of these muscles could pinch off the flow of mucus, then the food material could be freed in a sufficiently short time to account for the movement of the food. Such a muscular band has not yet been demonstrated, but the tentacles are provided with a good enough nerve supply throughout their length (Fig. 21, NT) so that such an arrangement should be well-coordinated. The third possibility does not require the presence of apical circular muscles, but merely the contraction of the longitudinal muscles and the depression of the central portion of the flattened papillar tip; this might serve to squeeze the openngs of the passages from the mucous-secreting cells and to stop the flow of mucus. Further studies are needed to elucidate the true nature of the mucus control of the papillae.

There is also some question as to the means whereby food material moves from the end of the papillated region of the tentacle to the mouth. The movement of the food is quick enough and smooth enough so that one might invoke a ciliated tract to explain this movement. However, there are no cilia whatsoever on the bases of the tentacles, on the ventral surface of the prostomium, or in the vicinity of the mouth. The coordinated movement of food material (in the case of *Artemia* eggs) moving down the tentacles with that inside the gut of the anterior region suggests that there is some connection between the two. It is possible that there is a mucus strand established at the beginning of feeding which bridges the gap between the papillated region and the mouth. This would serve as a "conveyor belt" which would insure that food went directly to the mouth; the observed identical oscillations of the food particles descending to the mouth and through the gut could well be caused by the muscular movements of the area of the gut which serve to draw the mucus strand and the adhering food posteriorly. This also merits further study.

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SUMMARY

1. Observations were made on the morphology, especially that of the circulatory system, the feeding, and the behavior of the polychaetous annelid *Magelona* sp. from the vicinity of Woods Hole, Massachusetts.

2. Features of particular interest related to the circulatory system include an anterior dorsal vessel which is divided into a series of chambers set apart by valves. The chambers undergo a precisely timed filling and emptying and pass blood forward to a heavily muscularized portion of the dorsal vessel whose contractions provide the primary pressure for movement of the blood. Segmentally arranged valves were noted in the posterior part of the dorsal vessel.

3. Burrowing is accomplished by the insertion of the prostomium into the substratum and the subsequent extrusion of the proboscis. This extrusion is brought about primarily by the hydraulic pressure of blood which has been expelled from the anterior region of the body by the contraction of all anterior somatic muscles except the proboscidial retractors.

4. *Magelona* sp. utilizes the papillae of its paired prostomial tentacles in feeding. Food material adheres to distal papillae and is transferred to more proximal papillae when a loop is formed by the tentacle; by repetition of this activity, the food material is passed stepwise toward the mouth.

5. Observations were also made on lateral organs, which may prove to be vibration receptors, and on so-called lateral pouches, whose function is unknown.

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CHEMOTAXIS IN THE CELLULAR SLIME MOLDS.
II. THE EFFECT OF CELL DENSITY

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The myxamoebae of the Acrasieae, or cellular slime molds, aggregate and form fruiting structures after they have ingested their bacterial food supply. The cell density must be above a minimum threshold before aggregates can be formed. This threshold varies in different species (Bonner and Dodd, 1962). As early as 1937, Arndt reported that larger fruiting structures develop at higher cell densities in *Dictyostelium mucoroides,* but their number remains constant per unit area. Also Shaffer (1962) pointed out the constancy of aggregation areas. Bonner and Dodd (1962) defined the areas covered by aggregates as aggregation territories and noted that these were specific for each species. In certain species a large variation in territory size occurs, but in *Dictyostelium purpurem,* the object of their study, the territory size is constant under various conditions and over a large range of cell densities (Bonner and Hoffman, 1963). Shaffer (1957) found that cells placed midway between aggregating streams at one side and other streams of different width, aggregation centers, or young fruiting bodies at the other moved approximately equally to both sides, despite large differences in the size of the acrasin-secreting sources. A quantitative method for studying acrasin secretion based on the attraction exerted by small populations of aggregating myxamoebae is described in this paper. The attracting myxamoebae were deposited at various densities in advance of the responding cells, and the minimal density for an optimal attraction per cell was determined. The results are discussed in the light of present hypotheses about cell aggregation.

MATERIALS AND METHODS

Spores of a haploid strain of *Dictyostelium discoideum,* NC-4(H), were inoculated with *Escherichia coli* #281 on a glucose-peptone medium (Bonner, 1947) and grown in darkness at 21–24 ° C. The cells were harvested in the late vegetative or early interphase stage in a 100 × diluted Bonner's salt solution (Bonner, 1947). Excess bacteria were removed by centrifugation. Small populations, which occupied areas with diameters of 300–700 microns, were placed on a specially prepared agar (Konijn and Raper, 1961) on which the cells remain inside the boundaries of the droplets. The concentration of the agar was reduced to allow myxamoebae to move outside the droplets when attracted by an external stimulus. The dishes, each containing about 160 small populations, were incubated in darkness at *ca.* 18 ± 1 ° C. The responding cells, which were deposited later, were stored at 5 ° C. in a full strength salt solution. The time interval between the deposition of the attracting and responding

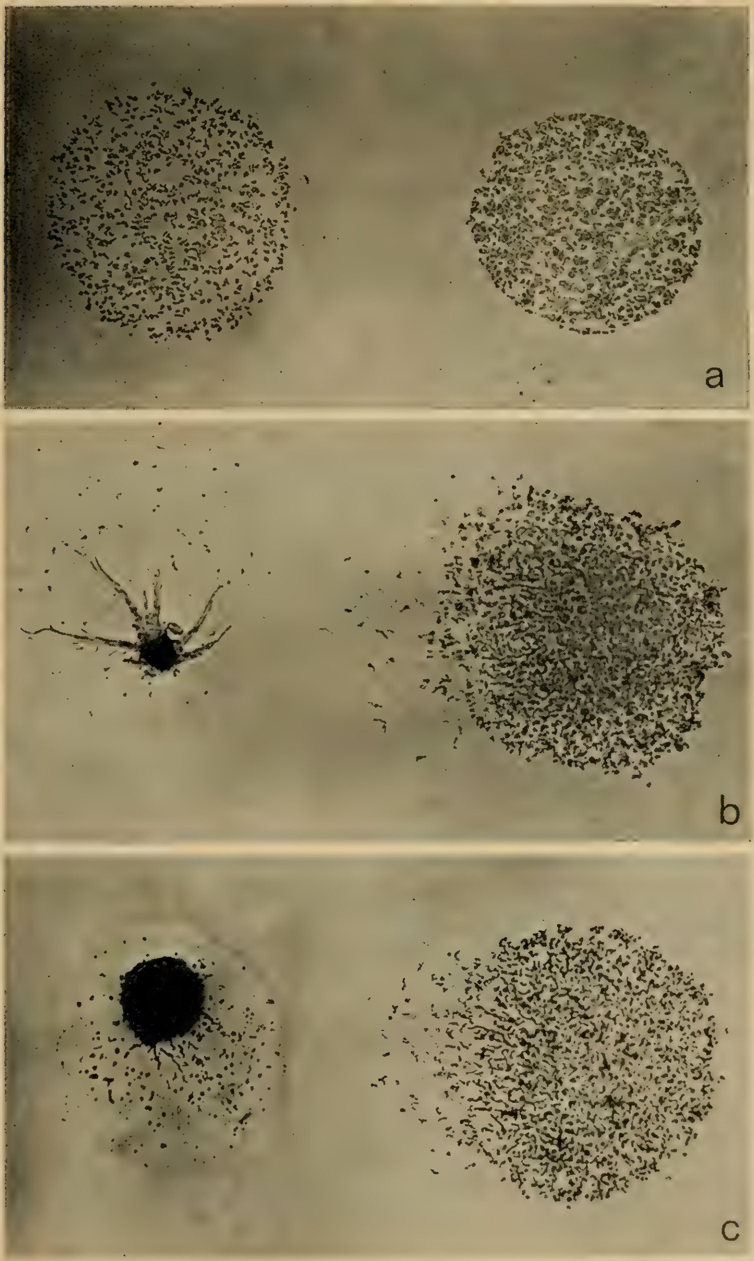

FIGURE 1. Small populations on hydrophobic agar of low rigidity. Attracting populations on the left side and responding populations on the right side. Magnification: × 60. a. Both populations are in the preaggregative stage. At this stage the myxamoebae remain inside the boundary of the drop. b. Myxamoebae in the responding population are attracted outside the boundaries of the original drop by an aggregation in the neighboring drop. c. The density of the myxamoebae in the attracting population was 6 times as high as in 1b. The chemotactic response, however, is not greater than in 1b.

populations was longer when the density in the attracting populations was lower. About 5½ hours elapsed after the deposition of the responding populations before the streams entered the aggregates in the attracting populations, and nearly all myxamoebae accumulated into a central mound of cells. The distance over which attracting myxamoebae were able to attract cells in responding populations was measured after the cells in the attracting populations had aggregated and before cells in the responding populations formed their own aggregates. The distance between the center of the aggregate in the attracting population and the nearest side of the responding population was measured through an ocular micrometer at a magnification of 80 ×. The attraction was marked positive only when cells in the responding drops moved outside the boundaries of the drop.

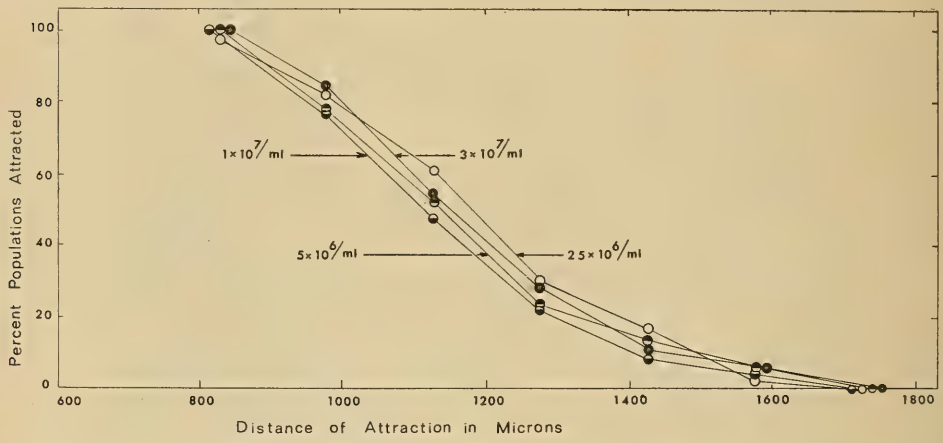

FIGURE 2. Four curves showing the percentage of responding populations that exhibit a response to developing aggregations in attracting populations derived from cell suspensions of different densities, and the distances between these aggregations and the nearest margins of the responding populations. The concentrations of the cell suspensions, from which the attracting populations were derived, are given. Diameter of the populations is 0.5–0.7 mm. Results represent the average of three experiments.

RESULTS

Washed myxamoebae were placed on hydrophobic agar of low rigidity to act as attracting and responding populations, and the cells comprising these stayed inside the boundaries of the droplets (Fig. 1a). When myxamoebae in the attracting populations aggregated, cells of the neighboring populations moved outside the margins of their droplets (Fig. 1b). The diameter of both populations was 0.5 to 0.7 mm. The attracting populations contained 500 to 1000 cells and the responding populations 2000 to 4000 cells. The myxamoebae of the responding populations, deposited several hours after the attracting populations, did not aggregate until after the attracting populations had already done so. Myxamoebae in attracting populations that varied 6-fold in density induced similar chemotactic responses (Fig. 1b *vs.* 1c). As a rule one aggregate per droplet was formed and all myxamoebae usually entered the aggregate.

The chemotactic response was virtually independent of the number of cells in the attracting populations over the range of 400–5000 myxamoebae. The cell suspensions from which these populations were derived contained $2.5 \times 10^6 = 3 \times 10^7$ cells/ml. (Fig. 2). The responding drops came from a myxamoebae suspension with 2×10^7 cells/ml. and contained 2000–4000 cells per population. All responding populations reacted positively at distances of 0.8 mm. or less. The distance over which cells in 50% of the responding populations were stimulated to move outside the droplet margins toward the attracting populations varied from 1.0 mm. to 1.3 mm. In a few cases sensitive myxamoebae were attracted over distances as great as 1.6 mm.

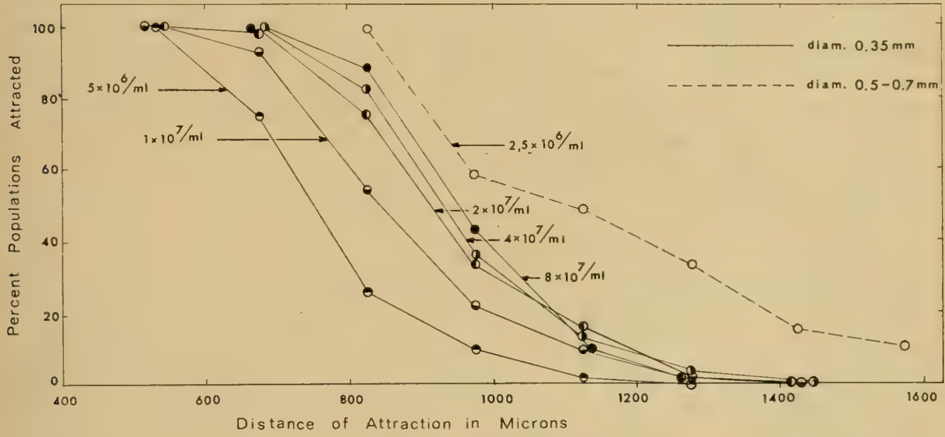

FIGURE 3. Four curves showing the percentage of responding populations that exhibit a response to developing aggregations in attracting populations of different densities, and the distances between these aggregations and the nearest margins of the responding populations. The concentrations of the cell suspensions from which the attracting populations were derived are given. The diameter of the droplets containing the attracting populations was *ca.* 0.35 mm. and that of the responding populations 0.5–0.7 mm. The diameter of the control populations (----) was 0.5–0.7 mm. Results represent the average of three experiments.

The delay before aggregation began varied with density. The onset of aggregation in populations with 5000 cells was *ca.* 5 hours after the deposition of the myxamoebae. Populations with *ca.* 400 cells started to aggregate after 8–11 hours. With still fewer myxamoebae the time of aggregation was considerably delayed.

Like the chemotactic response, the duration of aggregation was also independent of the cell density. The number of populations that had started to aggregate and the populations in which the streams had entered the aggregates were counted hourly. The time interval between the moment at which 50% of the populations started to aggregate and that at which 50% finished aggregation was 3–3½ hours. Control plates were kept in constant darkness. Attraction in these populations was similar to that in the plates that were exposed hourly to light.

No information was gained about the minimal number of attracting myxamoebae required for optimal attraction per cell under these conditions, because aggregation in populations of *ca.* 6 mm. diameter and less than 400 myxamoebae was strongly delayed. Since aggregates can be formed in small populations by far fewer cells (Konijn and Raper, 1961) the diameter of the populations was reduced to *ca.* 350 microns. The diameter of the responding populations and their density were the same as cited above. The myxamoebae suspension with the lowest concentration used contained 5×10^6 cells/ml. and the number of myxamoebae in the very small attracting populations deposited from this suspension varied from 90 to 180 cells. With these minute populations it was possible to show a decreased response when the cell density was low (Fig. 3). Droplets plated from a suspension with 5×10^6 cells/ml. attracted cells over a distance of *ca.* 0.75 mm. in 50% of the responding populations. A two-fold increase in the amoeba density resulted in a 50% attraction over 0.85 mm. Populations with 360 to 720 cells deposited from a myxamoebae suspension with 2×10^7 cells/ml. induced a positive response in 50% of the neighboring populations over slightly more than 0.9 mm. The minimal number of cells for maximal attraction was reached in these populations with a diameter of *ca.* 350 microns. Crowding the populations with two or four times as many cells did not result in an increased attraction.

Only attracting populations covering a larger surface area could extend the distance of attraction. The importance of the area covered by the myxamoebae is exemplified by comparing the attraction by populations with a diameter of *ca.* 0.35 mm. deposited from a suspension with 8×10^7 cells/ml. and populations with a diameter of 0.5 to 0.7 mm. deposited from a suspension with 2.5×10^6 cells/ml. (Fig. 3). The populations smallest in size contain *ca.* 2000 myxamoebae, or five times more cells than the larger-sized populations, but their attraction is 150 microns less. Also when the number of myxamoebae in attracting populations with different diameters was the same, the attraction by the larger population with lower density exceeded the attraction by the smaller population with the higher density.

The onset of aggregation was strongly delayed in some 0.35-mm. diameter populations derived from a myxamoeba suspension with 5×10^6 cells/ml. On the average, 13 hours were required before 50% of the attracting populations began to aggregate. Aggregation in the 0.35-mm. diameter populations with the greatest cell density started 7–9 hours after deposition on the agar.

The irregularity in time of aggregation in these very small populations of low density did not allow an accurate measurement of the duration of aggregation.

DISCUSSION

Sussman and Lee (1955) observed aggregative attraction in *Dictyostelium discoideum* through an agar membrane which was 0.205 mm. thick. Bonner (1947) noticed in the same species an orienting effect by underwater aggregates over a distance of 0.8 mm. Attraction over still larger distances was reported by Pfützner-Eckert (1950), who worked with *D. mucoroides,* but her results could not be confirmed (Shaffer, 1962). The results described in the paper show that centers of aggregation in *D. discoideum* are able to attract sensitive myxamoebae over a distance of 1.6 mm., and that only a small group of cells is required to achieve attraction over a maximal distance.

Bonner and Dodd (1962) measured the average radius of aggregation territories in different species. They found a mean radius of 1.27 mm. for territories in growth plates of *D. discoideum.* Aggregations formed by centrifuged cells covered a smaller area with a radius *ca.* 0.652 mm. A similar aggregation territory size was found by Konijn and Raper (1966) in dishes that were kept in constant darkness.

Bonner and Dodd (1962) suggested two possible ways of territory formation: the aggregation territory could be delineated by an inhibiting substance secreted by a cell at the center of the aggregation, or the boundaries of the aggregation were defined by the acrasin secreted by one cell or a small group of cells. The observed attraction over large distances favors the latter hypothesis, especially since even small groups of cells are able to attract over maximal distances. Shaffer, in 1962, assumed that there is no increase of acrasin production above a certain concentration by the whole group of cells, and a correspondingly reduced secretion per cell with an increase in the size of the aggregate. The mechanism responsible for the reduction of attracting substances per cell might well be a negative feedback.

The response of neighboring populations shown in Figures 2 and 3 was observed after they were exposed to chemotactic substances for a long period. Small aggregates, however, can also stimulate cells in responding droplets at a very early stage of aggregation, as has been shown in time-lapse films with attracting and responding populations in the same field (Konijn, 1965). When a fractional group of cells cooperated in center formation other cells in the attracting and in the responding populations were affected, independent of the cell density. Pulses produced at the onset of aggregation in the attracting population were transmitted through the agar to the responding populations, whose cells were attracted. This direct effect over large distances at the beginning of aggregation suggests that acrasin itself and not an inhibiting substance may be responsible for the size of a territory, although inhibiting substances may be present during aggregation.

Francis (1965) suggested that a steep acrasin gradient may prevent the formation of new centers in its vicinity, and Gerisch (1965) proposed that the pulses moving outward from a center of aggregation inhibit the formation of smiliar centers within its neighborhood.

The attraction by aggregates in very small populations is weaker than in populations with the same number of cells occupying a greater area. A higher density alone is not necessarily the cause of less attraction by minute attracting populations. It is possible that spatial relations may play a role. The populations that cover an area with a diameter of *ca.* 0.35 mm. may have shorter streams than larger populations with diameters of 0.5–0.7 mm. Since streams are also secreting acrasin (Runyon, 1941; Shaffer, 1957), larger populations with longer streams may show more attraction because the distance to the responding droplet has been measured from the center of the aggregate rather than from its periphery.

SUMMARY

1. The effect of cell density on chemotaxis in the cellular slime mold *Dictyostelium discoideum* has been examined by placing attracting and responding populations of washed myxamoebae at various densities on a hydrophobic agar surface. Aggregations in the former populations attracted cells out of neighboring responding populations which were deposited a few hours later. Attracting populations of 400–5000 cells, confined in droplets with a diameter of 0.5–0.7 mm., all induced a similar chemotactic response.

2. The period between early aggregation in the attracting populations and its completion was unchanging at the various cell densities.

3. Smaller populations with a diameter of *ca.* 0.35 mm. and containing *ca.* 500 cells gave a maximal secretion of attracting substance per cell when compared with cells at higher densities. At lower densities, the attraction by the whole aggregate was decreased. The results show that very small groups of cells optimally spaced are able to attract all myxamoebae within an area normally covered by an aggregation of *D. discoideum*.

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FUNCTIONAL MORPHOLOGY OF THE DIGESTIVE SYSTEM OF THE NUDIBRANCH MOLLUSC ACANTHODORIS PILOSA

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Since C. M. Yonge (1926a, 1926b) established the correlation between anatomy and physiology in mollusc digestion, a number of investigations on gastropod digestion have been reported (Graham, 1932, 1939; Fretter and Graham, 1962; Fretter, 1938–39; Howells, 1942; Carriker, 1946a, 1946b; Carriker and Bilstad, 1946). The significant publications on functional anatomy of nudibranch digestive systems are those of Graham (1938) on eolid digestion and Millott (1937a) on the sponge-eating dorid *Jorunna tomentosa*. The need for further basic studies of nudibranch digestion has led to this investigation of *Acanthodoris pilosa* (Abildgaard, 1789).

An early description of the anatomy of the digestive system of *A. pilosa* was that of Hancock and Embleton (1852). Subsequent to this, little reference was made to this species until the investigation and naming of its buccal pump by Forrest (1953). This dorid is a suction feeder, subsisting primarily on the ectoproct *Flustrellidra hispida*. The functional anatomy of its digestive system reflects modifications for manipulation of semi-liquid food. Investigations reported here of gross and microscopic anatomy, correlated with results of iron saccharate experimentation and ciliary current studies, elucidate the functions of the digestive tract of *A. pilosa*.

Appreciation is expressed to Dr. Wilbur Bullock for his direction and encouragement. Especial thanks are due to Dr. Melbourne R. Carriker for reading the manuscript and for his most helpful suggestions and criticisms.

MATERIALS AND METHODS

Nudibranchs were collected during the summers of 1963, 1964 and 1965 from an intertidal population at Edmunds on the northern coast of Maine. For immediate dissections, living animals approximately ¾″ to 1″ in length were relaxed in 8% magnesium chloride (w/v) and dissected in this solution. For gross dissection others were relaxed in 8% magnesium chloride, fixed 4 hours in Baker's formalin and stored in 1% propylene phenoxetol in distilled water (v/v). The latter method preserved some of the original colors of the organs and caused less shrinkage and distortion of the digestive organs than other methods.

Because of disagreement in the literature concerning the best fixative for nudibranch digestive organs (Millott, 1937a; Graham, 1938), a variety of fixatives including Bouin's, Bouin-DuBoscq, Helly's, Perenyi's, Baker's formalin, and AFA were used. Most useful was Bouin's fixative which gave less distortion of tissues than other fixatives. This is in disagreement with the conclusions of Millott (1937a) that this fluid is "relatively valueless." For cell inclusions, Helly's and Baker's formalin fixative proved best while Champy's fixative was good for cytological detail. Living animals were placed in containers of salt water resting in an ice-water mixture which served to decrease their activity. Under these conditions organs were excised and fixed in precooled vials of fixative. Tissues were routinely embedded in paraffin, sectioned at 7 μ and mounted on slides without adhesive.

Gomori's trichrome and Masson's trichrome were employed to provide general differentiation. These stains were especially valuable in distinguishing connective tissue from the epithelial lining of the digestive tract.

For nuclear detail in studies of cell types, Heidenhain's iron alum hematoxylin and Ehrlich's hematoxylin were utilized. Orange G, eosin and fast green served as counterstains.

Histochemical staining was employed to ascertain the chemical nature of the various cell inclusions. These stains included: Schiff reagent using periodic acid oxidation (PAS) with saliva and acetylation controls for carbohydrates, Best's carmine for glycogen, Mayer's mucicarmine for mucin, alcian blue and astra blau for acid mucopolysaccharides, and thionin and Mallory's phosphotungstic acid hematoxylin for carbohydrate metachromasia. For the general location of proteins the mercuric bromphenol reaction according to Mazia *et al.* (1953) was utilized; and for lipids, oil red O was employed on frozen formalin-fixed material. Additionally Von Kossa's method for calcium and Gomori's alkaline phosphatase methods were utilized.

Ciliary currents were studied with a suspension of animal charcoal dropped in the desired area of the predissected digestive tract. Pathways shown by movement of particles were observed under a dissecting microscope and recorded on pre-drawn outlines of the digestive tract.

Nudibranchs to be utilized for the iron saccharate experiments were starved for 24 hours and then relaxed in 8% magnesium chloride (w/v). A suspension of iron saccharate was injected into the mouth cavity by means of a medicine dropper. At intervals of $\frac{1}{2}$, 1, 3, 6, 10, and 16 hours, experimental animals were fixed in equal parts of Bouin's fixative and a 5% solution of ammonium sulphide in 95% alcohol. The digestive tract of fixed whole animals was excised, embedded, and sectioned. Tissue sections were stained for the Prussian blue reaction (Fretter, 1937) and examined for distribution of iron saccharate. As a control, an animal not injected with iron saccharate was fixed in the prescribed mixture and examined for the natural occurrence of iron.

OBSERVATIONS

The digestive system of *A. pilosa* is divided into four functional areas. The most anterior one is formed by the mouth, buccal cavity and associated parts, and functions in reception of food. Conduction and storage take place in the next area of the digestive tract which includes the esophagus and an expanded portion, the crop. The latter is followed by the stomach, digestive gland and connecting ducts which form the area concerned with digestion and absorption. Next come the cecum and intestine which are principally concerned with formation of feces.

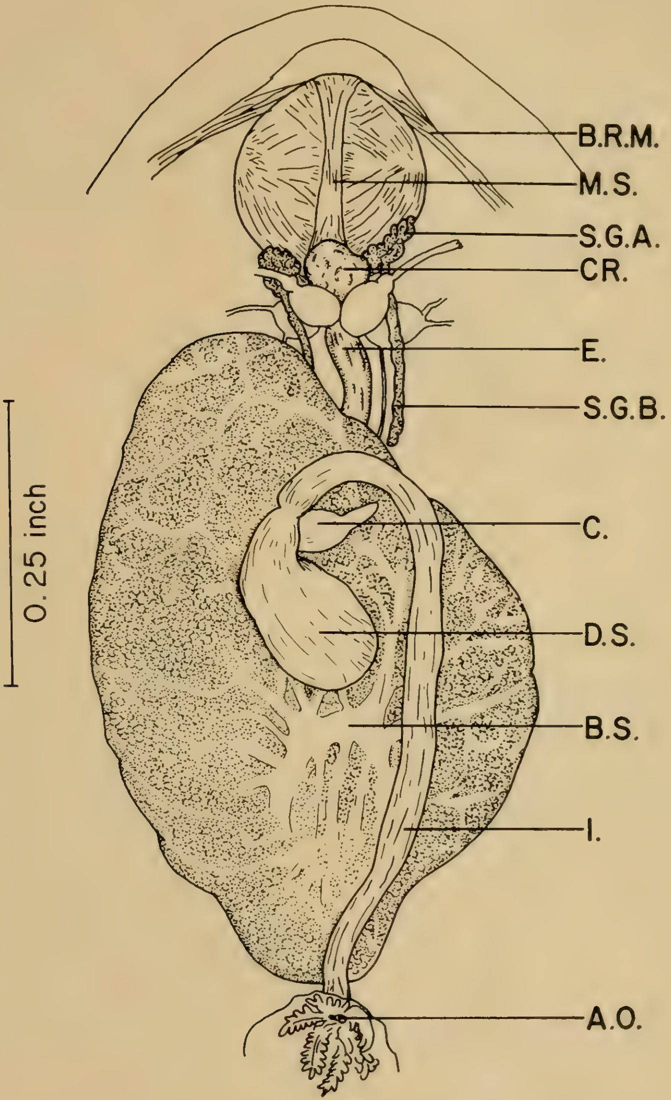


FIGURE 1. Gross morphology of the digestive organs of *A. pilosa* exposed by cutting through the dorsal mantle and tough thin membrane coverings. Drawn from nudibranch fixed in Baker's formalin and stored in 1% propylene phenoxetol. A.O., anal opening; B.R.M., buccal retractor muscle; B.S., blood sinus; C., cecum; CR., crop; D.S., dorsal stomach; E., tubular portion of esophagus; I., intestine; M.S., median strip; S.G.A., follicular portion of salivary gland; S.G.B., thin flattened portion of salivary gland.

Region of food reception

The mouth is anterior to the foot and is separated from it by a transverse furrow. Surrounding the mouth are the oral tentacles. These are modified to form a veil and are drawn out on either side into muscular leaf-like processes. During the probing activity of the animals, these processes are rounded or acutely angled. In preserved nudibranchs the processes appear triangular and ridged. Around the lateral and anterior border of the veil is a grooved depression caused by the overhang of the mantle. Thus the plane of the mouth, because of the position of the fleshy veil, is the same as that of the foot, and when the animal is crawling, is in continual contact with the substrate. The mouth appears as a longitudinal slit surrounded on three sides by the fleshy veil.

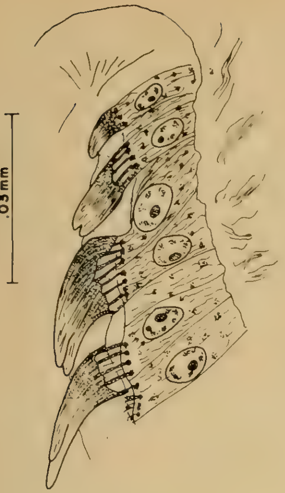
The epithelium of the oral veil is ciliated columnar with unicellular and multicellular glands in the subepithelial area. Ducts pass from the glands through the epithelium and empty their secretion on the surface (Fig. 6).

When this outer lip is cut away a short tubular connection to a ridged inner lip is revealed. This short channel is also lined by ciliated columnar epithelium and the subepidermal space is abundantly supplied with multicellular glands which add more secretion to incoming food. The nuclei are located near the outer borders of the gland cells and the cytoplasm is filled with a secretory substance which stains metachromatically with thionin and orange with safranin O. The epithelium of the inner lip is secondarily folded and on the medial and inner surface is covered by a homogeneous cuticular layer. The submucosa contains numerous muscle fibers scattered in loose connective tissue.

Beyond the inner lip is the non-ridged smooth-surfaced buccal lip characterized by numerous minute, closely set, bifid spines. The epithelium underlying the spines varies in shape from cuboidal to columnar and the cytoplasm of the cells in the inner groove of the buccal lip is basophilic. Within these cells are numerous well defined canaliculi and near their outer border are concentrations of secretion associated with the canaliculi. The latter appear to continue through the border of the cell and pour their secretion into the base of the spine which caps one or two cells (Fig. 2).

The buccal mass (Fig. 1) occupies the anterior fourth of the oval visceral area. The dorsal portion is composed of a bilobed bulb divided mid-sagittally by a white tough strip of muscle. On either side of the median strip, muscle fibers radiate laterally and symmetrically forming the thick-walled buccal pump (Forrest, 1953). On the ventral surface of the buccal pump is the anterior opening into the digestive tract and just behind the spined buccal lip are a pair of hard rudimentary plate-like jaws.

The opening of the esophagus is on the posterior dorsal surface of the buccal mass and immediately below this from the posterior ventral surface the radular sac protrudes. On either side of the esophagus and emptying into the dorsal lateral walls of the buccal mass are paired salivary glands. The glands are divided morphologically into two portions, a proximal follicular portion and a thin, distal flattened, string-like portion. Histologically they do not appear differentiated, and both parts are characterized by large cuboidal epithelial cells which stain strongly basophilic and have rounded nuclei with distinct nucleoli. The duct from the gland



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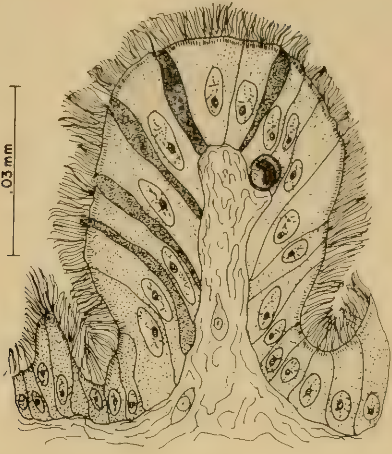

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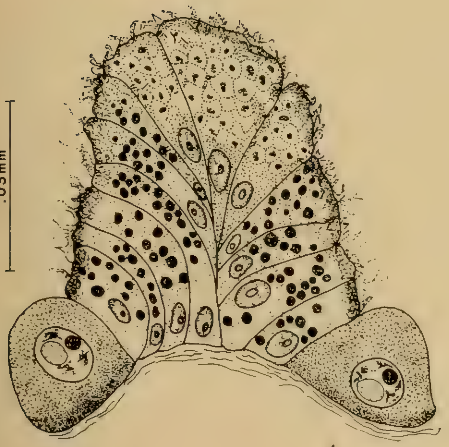

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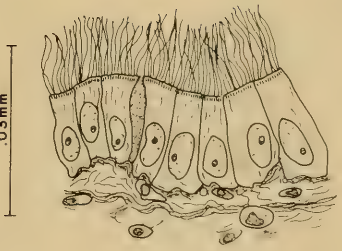

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FIGURES 2–5.

FIGURE 2. Portion of a section through the groove along the inner edge of the buccal lip. The intracellular canaliculi pass from the epithelial cell into the buccal spine capping the cell. Camera lucida drawing from Bouin's-fixed material.

FIGURE 3. Section through the digestive gland duct of *A. pilosa.* The thin secretory cells are dispersed among the ciliated columnar cells of the ducts. A wandering cell with its characteristic bean-shaped nucleus is in the epithelium. Camera lucida drawing from Helly's-fixed material.

FIGURE 4. Section through a fold of the digestive gland of *A. pilosa.* The large round granules are associated with Type B tubule cells. Type A tubule cells are vacuolated at the distal ends. The crypt cells are characteristically triangular-shaped in section. Camera lucida drawing from Perenyi's-fixed material.

FIGURE 5. Portion of a longitudinal section through the intestine of *A. pilosa.* Camera lucida drawing from Bouin's-fixed material.

to the buccal cavity is lined by a thickly ciliated epithelium, and the entire structure is covered by a thin layer of connective tissue.

Bisection of the buccal mass reveals strong muscular walls in the buccal pump region leaving a small cavity inside. The columnar epithelium of the interior is covered by a thick cuticle which is differentiated into an acidophilic layer proximal to the cell border and a distal uneven basophilic layer. Projecting into the anterior cavity of the buccal mass from the floor is the bilobed odontophore with the toothed radular ribbon in the median groove. During feeding the odontophore is protracted, spreading the radula at the bending plane for rasping. The cuticularized lining of the anterior part of the buccal cavity and rudimentary jaws forms a protective shield over the underlying epithelium. The radula disappears posteriorly into the radular sac.

The epithelium in the dorsal region of the cavity of the buccal pump is invaginated to form a groove along the median line which deepens as it passes posteriorly toward the esophagus. Glandular epithelium lines the walls of the buccal cavity lateral to the ciliated dorsal groove and above the odontophoral muscle connections to the buccal wall. The cells are distended by a secretion that is metachromatic and PAS-positive. It is in this dorso-lateral area that the salivary gland ducts enter and thus both secretions mix with the food.

Area of conduction and storage

The opening connecting the buccal cavity with the esophagus is on the posterior dorsal surface of the buccal mass. The esophagus enlarges dorsally into a thin-walled crop (Fig. 1). In dissections of living animals, the crop is either collapsed or rigidly distended with a liquid material. The posterior portion of the esophagus is of a uniform diameter and passes directly back from the buccal mass, disappearing into the mid-anterior surface of the digestive gland.

At the area of transition from buccal mass to esophagus, the dorsal ciliated groove forms approximately 11 small, ciliated ridges which coalesce into six major ridges and lead into the crop. There are two valve-like invaginations of the epithelium at the entrance of the crop. The crop epithelium is ciliated and there are irregular patches of glandular epithelium throughout. When the crop is distended, it is difficult to delineate cell boundaries. The posterior portion of the esophagus is tubular and lined by ciliated columnar epithelium. There are rounded glandular epithelial cells scattered throughout the tubular portion.

Region of distribution, digestion and absorption

The relationship of the stomach and digestive gland is apparent in dissections and reconstructions from serial sections. The tubular esophagus enters the anterior face of the large heart-shaped digestive gland (Fig. 1) and empties onto the floor of the stomach. This portion of the stomach is thin-walled and receives the two major ducts from the digestive gland. These communicate with a series of minor ducts and lead to the blind tubules of the gland. Numerous blood vessels envelop the organ and penetrate through the connective tissue.

An exposed muscular portion of the stomach lies in a depression on the mid-dorsal surface of the gland (Fig. 1) and has a highly folded mucosa enveloped by circular muscle. The intestine emerges from this exposed region.

A layer of ciliated columnar epithelium lines the thin-walled floor of the stomach cavity. The cells of the epithelium vary in height, with low cells in the crypts and tall columnar cells on the ridges. A glandular area extends along the upper wall above the duct openings. Columnar cells bearing cilia are wedged in between the secretory cells.

The major digestive gland ducts are lined with tall ciliated columnar cells arranged in distinct longitudinal folds (Fig. 3). There is a well delineated ciliated border with thin exocrine glands dispersed in the epithelium. The glandular cells are more numerous and rounded droplets of secretion are often present in the lumina of the more distal ducts.

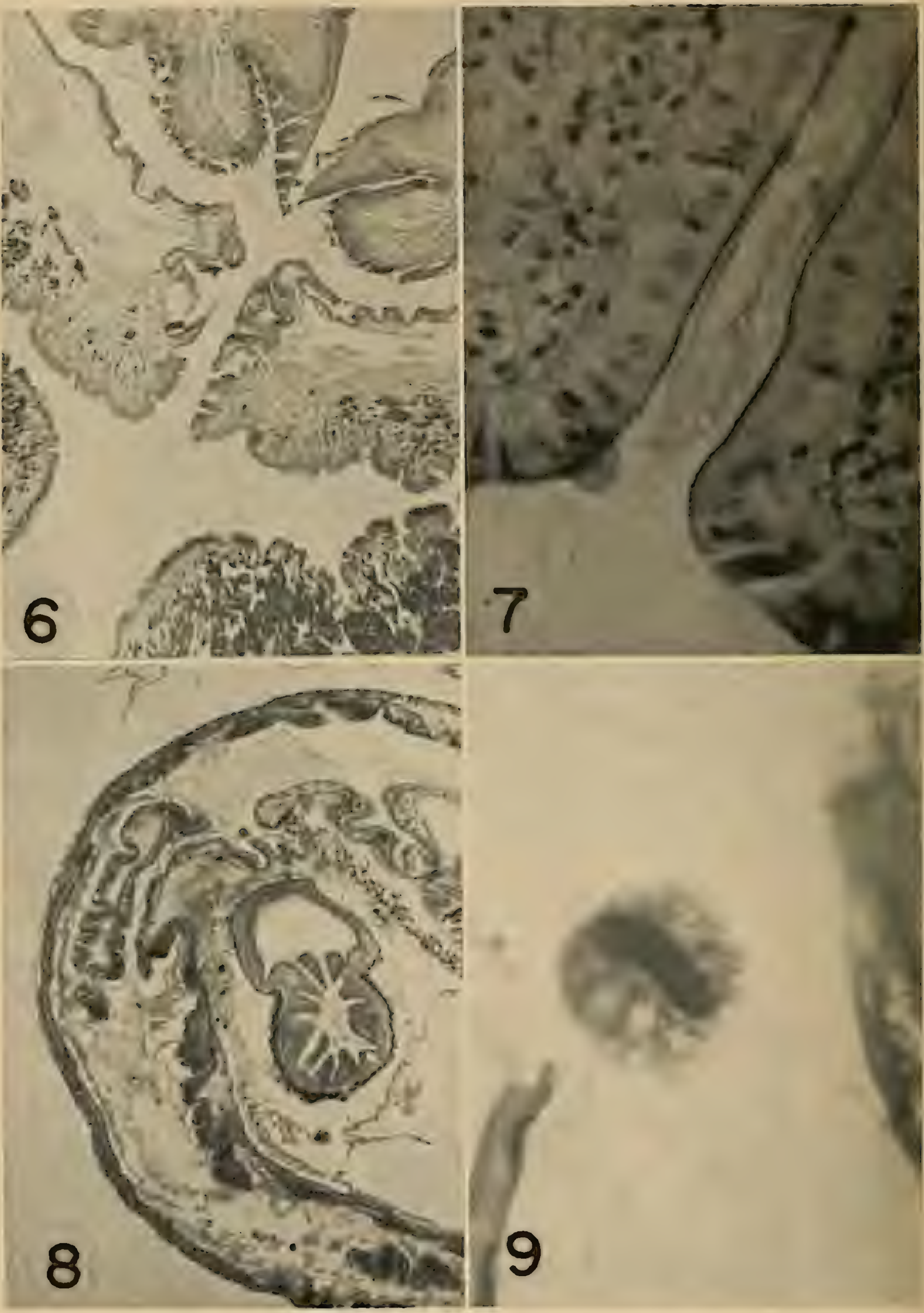
The mucosal folds of the dorsal portion of the stomach are pronounced and project into the reduced lumen. Major folds lined with ciliated columnar epithelium are secondarily ridged, exposing a large amount of cellular area to the lumen. This portion of the stomach is distinguished from the ventral area by the increase of connective tissue in the submucosa and the well developed surrounding circular muscles.

The epithelium of this digestive gland is differentiated into two general cell types, the crypt cells and the more numerous tubule cells. The crypt cells are named on the basis of their characteristic position within the digestive gland. They are triangular in section and are located in the crypts of the folded epithelium with the broad base of the triangle resting on the basement membrane. There are several of these cells adjacent to each other in the crypts. The nucleus is characteristically large and spherical with a prominent rounded nucleolus surrounded by chromatin in a reticular pattern. With the majority of fixatives the cytoplasm of the crypt cells is homogeneous and has a strong affinity for basophilic dyes. The cells do not show any concretions, granules or other detectable cytoplasmic inclusions.

The most abundant cells lining the gland are the tubule cells. They are club-shaped in appearance, form the invaginated folds of the tubules and have basal nuclei. The nuclei are oval in shape with discernible nucleoli and very little chromatin in the nucleoplasm. Tubule cells are differentiated into tubule cell A and tubule cell B due to the characteristic cell inclusions, the height of the cells and their relative position in the epithelium (Fig. 4).

Tubule cell A is generally located on the inner edge of the epithelium, is narrow at its attachment to the basement membrane, and broadens as it projects into the lumen of the tubule. The free border of the cells varies in shape and often appears in different stages of constricting "fragmentation phagocytes" (Morton, 1955) into the lumen of the tubule. In these cases just beneath the outer border of the cell lies a layer of homogeneous basophilic cytoplasm bordered by an area packed with small closely set vacuolar-like spaces. The spaces vary in their content of particulate matter. Glands fixed in Helly or Susa fluids show an area of light brown fine granulation in the middle and upper portions of the tubule cells A. Evidence from squash preparations indicates that the pigment of these granular masses produces the light brown color of the living digestive gland.

The type B tubule cells differ in morphological position and granular inclusions. Type B cells are found along the sides of the digestive gland folds between type A tubule cells and the crypt cells. They are slightly narrower at the base and



Figures 6–9.

not as tall as type A cells but resemble them, being columnar and having basal nuclei of similar staining reaction and shape. These cells are characteristically filled with distinct round granules that stain bright red with Masson's trichrome, blue with Mallory's phosphotungstic acid hematoxylin and deep blue with azure eosinate at pH 6.0. In several sections the granules were seen in the lumen of the gland, digestive gland ducts and the intestine.

In the distal two-thirds of both tubule cells A and B it is common to find large vacuoles which often distort the cell outline. These vacuoles contain one to four rounded spherules. These are conspicuous in formalin-fixed material and are somewhat shrunken after Helly's fixative. Frequently these spherules were seen in the gland ducts and in the lumen of the intestine, appearing to have broken out of their vacuoles completely.

In the underlying connective tissue the most numerous cells are the amoebocytes containing very little cytoplasm and nuclei with an intense affinity for nuclear stains. The amoebocytes are most numerous just beneath the mucosa of the digestive gland ducts and are especially prominent in glands stained with iron-alum hematoxylin. There are oval lightly-staining nuclei under the epithelium which are associated with the reticular and fibrous strands of cytoplasm and may represent the nuclei of drastically shrunken Leydig cells of the connective tissue. Numerous blood cells are associated with the connective tissue.

The contents of the lumen of the digestive gland are variable. Fragmentation phagocytes are most frequently observed in glands where tubule cell A is drawn out into a long thin cell with a vacuolar top. This poses the question as to whether the observed bodies in the lumen are due to the sectioning procedure or whether they are truly separated phagocytic bodies abstricted from the tips of the type A tubule cells. Evidence of complete separation of these bodies supports opinions of Millott (1937a) and Morton (1955). Serial sections show that these bodies are not connected in all cases to the tubule cells. Cells are often found in the lower part of the stomach and in the digestive gland ducts where there is no digestive gland epithelium present. The cells have been observed in various stages of abstricting from the digestive epithelium and the vacuolar nature of the cytoplasm is still evident in the phagocytes when they are found in the lumen. The periphery of the fragmentation phagocytes has a layer of basophilic cytoplasm just as is found at the tip of the tubule cell before abstriction. The presence of these phagocytes is considered to be a natural occurrence and not the result of sectioning.

Spherules which occur in the large vacuoles in both tubule cells A and B are

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FIGURE 6. Transverse section of the inner lip and spined buccal lip of *A. pilosa*. The multicellular glands are shown darkened with stain and distributed in the subepidermis on the inner side of the oral veil and the outer edge of the inner lip. Fixed in Bouin's, stained with safranin 0 and fast green (× 60).

FIGURE 7. Section through the anal opening of the digestive tract of *A. pilosa*. Fixed in Baker's formalin, stained with hematoxylin and eosin (× 600).

FIGURE 8. Section through the U-shaped junction of the stomach and intestine with the cecum located in the bend. Fixed in Bouin-DuBoscq, stained with Gomori's trichrome (× 400).

FIGURE 9. Wandering cell in the intestine of *A. pilosa* displaying the characteristic bean-shaped nucleus and vacuolated cytoplasm. Fixed in Baker's formalin, stained with hematoxylin and fast green (× 900).

also encountered in the lumina of the digestive tubules. They are often found in conjunction with the round granules from tubule cell B.

Region of formation of feces

This area includes the tubular intestine and the cecum. The latter is an evagination of the dorsal stomach wall at the junction of the stomach and intestine.

The cecum is lined with tall ciliated columnar cells, variable in height and forming finger-like projections into the lumen (Fig. 8). The ciliated border is indistinct and the cilia project far into the lumen. The epithelium rests on a layer of circular muscle.

The ciliated columnar epithelial cells of the intestinal mucosa are relatively uniform in size and shape (Fig. 5). The epithelium is arranged in distinct longitudinal folds which do not change in size appreciably until the anal region. Here they deepen and the cells become regular in outline (Fig. 7). Glandular epithelial cells are present throughout the intestine and the submucosal glandular areas are prominent at the anal region.

Discussion

Although *A. pilosa* may feed on a variety of foods, it is usually found closely associated with *Flustrellidra hispida* and has been observed feeding on this ectoproct. During feeding the oral veil is appressed tightly to the substrate. The odontophore is projected through the cuticularized rudimentary jaws and the radula scrapes the surface of the ectoproct zooecium. The spined buccal lip may serve to anchor the animal to the ectoproct during rasping. Once a hole is rasped, the buccal pump withdraws the soft contents from the prey into the buccal cavity. Along the way secretions are added from the oral tentacles and the short tubular channel to the inner lip. Material is passed to the dorsal ciliated groove where it mixes with secretions from the glandular buccal wall and salivary glands. Whether the salivary glands produce enzymes has not been determined. Although the animal does not take in large amounts of zooecia, small pieces as well as numerous diatoms have been found in various regions of the gut.

The crop serves to store large quantities of food during prolonged periods of feeding. At the entrance a valve controls passage of food into the crop. The semiliquid food appears homogeneous and contains a minimum of particulate matter. No diatoms or pieces of zooecia were found. This indicates these materials may bypass the crop through the heavier ciliated tubular portion of the esophagus. The latter is folded longitudinally and varies in appearance depending on the amount of food present. The cilia play an important role in conducting food from the esophagus onto the floor of the stomach.

Ciliary currents indicate the food is immediately distributed to the large major digestive gland ducts leading eventually into the tubules of the digestive gland.

It has been established that there is a rhythmic cycle of secretion, digestion and absorption of food materials in the digestive tract of molluscs (Krijgsman, 1928; Thiele, 1953). This may account for the variety of cellular inclusions in the digestive gland, and the past confusion in the literature regarding cell types. Once food is taken into the digestive tubules, tubule cells become elongate and club-shaped especially in cell type A, near the lumen of the tubule. In animals 10 and 16 hours after introduction of iron saccharate, there were numerous large fragmentation phagocytes with distinct particles of iron in the vacuoles. This suggests an initial ingestion of particulate matter into the rounded tips of the tubule cells followed by an abstriction of the fragmentation phagocytes into the lumen. The iron saccharate experiments were not designed to determine timing of the cycle, but results indicated a correlation between the fragmentation phagocytes and a phase of intracellular digestion. Abstriction of these fragmentation phagocytes provides an increased surface area for ingestion of material for intracellular digestion.

It is difficult to ascertain whether any zymogen granules are given off by the tubule or crypt cells during the digestive cycle. Present studies indicate that the main type of digestion in *A. pilosa* is intracellular but does not exclude the possibility that enzymes are secreted by the salivary glands or the cells of the digestive gland. In alkaline phosphatase experimental sections activity is indicated at the border of the crypt cells and tubule cells of the B type. This may be due to movement of secretion molecules across the cell border. Thiele (1953) has suggested that pre-secretory granules are present during the feeding cycle of *Helix* and that the secretion binds with protein in such a way that it is undetectable in stained sections. Yonge (1931) mentioned that a phylogenetic development of extracellular digestion in gastropods is correlated with a change from an herbivorous diet to a carnivorous diet. This is yet to be shown for *A. pilosa.* Due to the watery nature of food consumed in the diet, there appears to be a retention of intracellular digestion and lack of specialization of the gut to produce elaborate enzymes for extracellular digestion of grossly particulate foods.

The role of the crypt cells in the digestive gland of *A. pilosa* is not clear. Similar cells contain calcium spherules in many pulmonates and some prosobranchs. Wagge (1951) and Abolins-Krogis (1960) have indicated their importance as a supply of calcium for shell repair. In pulmonates the calcium is transported by blood cells to the site of repair. In *A. pilosa* there is no indication of the presence of calcium which can be correlated with the absence of a shell.

Crypt cells have been shown to be areas of storage (Wagge, 1951). This function may occur in *A. pilosa,* since the cells stain positively for protein material with brom-phenol blue and are slightly PAS-positive. Additionally there are indications of alkaline phosphatase activity at the cell borders due possibly to absorption of digested material.

A part of the rhythmic digestive cycle of *A. pilosa* involves "extrusion" (Forrest, 1953) of a variety of cell inclusions. The digestive epithelium during this period appears irregular with protoplasmic strands extending from the borders, and a variety of inclusions are present in the lumina of the tubules, stomach and intestine. There are four types of material which appear to be extruded. The exact nature of these materials and their origin are not understood. All four materials appear together in the lumina of the tubules in a single gland. Furthermore, similar granules are found in fecal strings given off by living animals.

The round granules typically associated with tubule cell B, which stain bright red with Masson's trichrome and blue with Mallory's phosphotungstic acid, are also found in the lumina of the digestive tract. In fecal strings from living animals, granules of approximately the same shape and size appear reddish in color. Just what these granules are is not known. Abolins-Krogis (1960) considered similar tubule cells to be excretory cells. She maintained that these cells and the digestive cells (corresponding to cell A) were variants of the same cell type.

The granular material associated with tubule cell A, which is so evident in Helly- and Susa-fixed material, is also considered a waste material. The evidence for this is not complete. The coloration of this material is yellow-brown and it is egested in the feces.

Small rounded, somewhat vacuolated cell fragments are often encountered in intestinal contents. It is suggested that these are the end products of the fragmentation phagocytes. The phagocytes have completed their breakdown of food materials, the soluble materials are given off into the lumen and in some way reabsorbed by the lining cells, and the spent fragment of a cell is egested.

Finally, the large yellow round concretions of the large vacuoles in the tubule cells are also found in the lumina of the tubules, stomach and intestine, as well as in fecal strings of living animals. Regarding these concretions in prosobranchs, Fretter and Graham (1962) state (p. 230) "Their real nature is unknown, but as they may often be found, apparently unaltered, in the faeces, they appear to be excretory matter of some sort." In prosobranches and opisthobranchs these concretions are formed in different cells. In some prosobranchs (Fretter and Graham, 1962) they are associated with the large "triangular-shaped cells" (crypt cells in *A. pilosa*). In *A. pilosa* they are confined to the tubule cells and have not been observed in the crypt cells. Their shape is variable, due perhaps to their stage of formation. In some cases they are in a concentric ring form, indicating formation by accretion. Cells containing similar granules were extensively pictured by Henneguy (1925) and he stated that the particles were composed of xanthin, a waste product of nitrogenous metabolism.

The digestive gland is a complex site of digestive processes. The complexity is increased by the reaction of the cells and their contents to different fixatives. These problems, along with the obvious rhythmic cycle involving secretion, absorption and extrusion, render it difficult to establish a well defined description of the gland for any one animal. Previously a variety of names and functions has been assigned to the cells of the digestive gland. On the basis of present knowledge of *A. pilosa* it is impossible to designate cells of the digestive gland tubules as either digestive or excretory. Until further studies on enzymes and the feeding cycle are carried out, these cells are tentatively named tubule cells (variants A and B) and crypt cells.

There are two blood cells that are associated with various organs throughout the digestive system. One is a small amoebocyte generally located in the connective tissue. They are most numerous in the submucosa of the digestive gland ducts. In some, the small cytoplasmic areas show a positive glycogen reaction to Best's carmine stain. These amoebocytes may play a role in the transfer of food materials from the fragmentation phagocytes to the crypt cells for storage as they are often numerous at the edges of the crypt cells in the digestive gland.

The other type of cell is the wandering cell (Fig. 9). Millott (1937b) discussed the occurrence of wandering cells in the digestive epithelium of a variety of nudibranchs including *A. pilosa.* Utilizing the Prussian blue reaction, he injected iron saccharate into the haemocoel. By means of fixation at intervals and studying sections of the animals, he studied the subsequent distribution of iron. He concluded these cells were wandering cells, amoeboid when living, and that they were actually a special type of blood cell. He further stated (p. 412) "evidence indicates that the wandering cells of nudibranchs are excretory, taking up effete matter from the haemocoele and discharging it into the lumen of the gut." In *A. pilosa* these cells were observed throughout the lumen of the gut. They are rare in the esophagus and most numerous in the intestine and cecum. They are capable of moving freely between the connective tissue of the submucosa and the lumina of the organs and in several cases were seen passing between the epithelial cells. Millott (1937b) suggested that the cells discharged their products into the lumen of the gut and then migrated back through the wall. Observations in *A. pilosa* confirmed the wandering behavior of these cells although the nature of their inclusions is not known. The cells are not seen in the blood sinuses. This may be due to a different staining reaction of the cell when it is being formed, or there may be a different origin of the cells in *A. pilosa.*

It is difficult to understand how extrusible material in the digestive gland moves into the stomach cavity. Millott (1937a) described the cilia of the ducts as beating both in the direction of the blind tubules and toward the dorsal stomach in separate tracts. This was not seen in *A. pilosa* although this might have been due to the clumping of the carbon particles rendering a reverse current invisible. It would seem logical that the contents of the tubules are caught in an ascending current and swept into the muscular stomach.

Ciliation of the hindgut is similar to that of the tubular esophagus. There are major tracts in the intestine which conduct the feces to the outside. Because of thickening of the folds at the anus, the feces are ejected forcefully from the opening. The feces come out in spurts which aid in prevention of fouling of the branchial area.

Yonge (1931) has suggested that in gastropods there is a correlation between the compactness of the fecal string and the position of the anus relative to the mantle cavity. Prosobranchs which have undergone torsion have elaborate mechanisms which compact undigested material, thus preventing fouling of the closely associated mantle cavity. Opisthobranchs on the other hand have undergone detorsion and the anus is located on the postero-dorsal surface. In *A. pilosa,* feces drop off the sides of the animal, the fecal string is not well compacted and the cecum is reduced in size. The functional morphology of *A. pilosa* supports Yonge's theory.

The cecum in *A. pilosa* is a small structure which communicates with the stomach. A ciliated groove leads into the narrow opening of the cecum. In one animal, a fecal string was found in the intestine which consisted of compacted detritus surrounded by a thick clear layer of mucus. Recognizable in the pellet were many wandering cells. This was not seen in the majority of animals; the cecum is generally devoid of any material and only occasionally are diatoms and pieces of teeth found in the intestine. The glands in the cecum give rise to the mucus which covers the large particulate waste materials and the cilia function to compact any pieces of hard parts into small pellets. The rare presence of hard parts of the animal's food would account for the small cecum. Millott (1937a) reports a well developed cecum in the sponge-eating dorid *Jorunna* *tomentosa.* In this instance the cecum serves to coat sponge spicules with mucus and compact them into a fecal string. The cecum of *A. pilosa* only forms small fecal pellets.

SUMMARY

This investigation has demonstrated that functional anatomy of the digestive system of *Acanthodoris pilosa* reflects the following modifications for manipulation of semi-liquid food:

1. The anterior buccal mass is enlarged forming a muscular buccal pump which is utilized to suck the contents of the ectoproct into the buccal cavity once the radula has rasped a hole in the zooecium.
2. A spined buccal lip at the entrance of the buccal cavity probably functions to anchor the muscular buccal mass during pumping and/or scraping activities.
3. An enlargement of the antero-dorsal wall of the esophagus forms a thin-walled crop capable of distension and functioning for storage of semi-liquid food.
4. The digestive gland epithelium consists of two cell types, pyramidal-shaped crypt cells and more numerous tubule cells with their variants A and B. The rhythmic nature of the digestive epithelium involves: (1) a period of ingestion of food particles by fragmentation phagocytes and the tips of the tubule cells, (2) a concentration of undigested materials in the tubule cells, and (3) extrusion of these waste materials into the lumina of the digestive gland.
5. There is a reduced cecal evagination of the dorsal stomach which probably functions to form small fecal pellets.

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HEMATOLOGICAL RECOVERY OF MOUSE FOLLOWING FETAL X-RADIATION

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Of all of the tissues studied blood is probably the most sensitive to exposure to any of the ionizing radiations. Changes in blood cell counts can usually be relied upon as diagnostic for a recent exposure. While the lymphocyte appears to be the most sensitive of the cellular elements, most of the cells of the blood of the adult or of the fetus are radio-responsive (Hulse, 1963).

Hematopoiesis is very active in the mouse embryo at 15 days' gestation, but blood counts are difficult to obtain before gestation day 17. It seemed of interest to x-radiate the 15-day mouse fetus with graded doses of x-rays and then to determine the response of the blood elements as determined 2 days later, at gestation age 17 days. This is the earliest stage at which fetal blood can be analyzed satisfactorily. A further analysis was made at 3 weeks of age or at the time of weaning.

Materials and Method

The CF1 white mouse was used exclusively for this study, the 15-day fetuses being x-rayed *in utero* and the 17-day fetuses being removed for blood analyses. Others were born normally and kept with their mothers for three weeks.

Mature females of this strain were time-mated from 8:00 AM until 10:00 AM and those with vaginal plugs (indicating successful mating) were isolated and labelled. At exactly 15 days thereafter 20 pregnant females were x-rayed in groups of five, equidistant between parallel x-ray tubes at 72 cm. distance from the gravid uteri. Filtration of 0.28 mm. Cu and 0.50 mm. A1 hardened the rays; the HVL was 0.6 mm. lead and the KVP 184 and MA 30. The output was 50 R/min. For the low dose of 5 R the conditions were different, since there was a 2-second build-up of the output which was included in the prior dosimetry. The modification included an exposure of 8 seconds which, according to the Victoreen check, was exactly 5 roentgens in air at the position of the uteri.

Blood was always taken before noon of the day designated in order to avoid any diurnal shift in the normal blood counts (Urushiyama, 1959). From the 17-day fetus blood was taken quickly with pipettes and rubber tubing all ready. The head of the fetus was held in the left hand, the lower part of the abdomen being immobilized by the thumb of the same hand. The jugular vein is plainly visible through the thin fetal skin, and this was pierced with scissors. All counts were based upon samples from 10 mice, fetal or post-natal.

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At 3 weeks of age the blood was taken by snipping off the end of the tail and allowing the blood to emerge. Occasionally it was necessary to take blood from a second fetus, but each count represents blood from different fetuses. It was not feasible to select for sex, but with averages from 10 fetuses (or more) selected at random any sex variables would be cancelled out. On several occasions, especially in x-rayed mice, the amount of blood was inadequate so that half volumes were taken, and the counts doubled since by dilution the final counts would be half the absolute values.

Experimental Data

The exposure of mouse fetuses at 15 days' gestation to doses of 100 R or less has little effect on their survival to birth. (It takes 750 R to kill 50% of such fetuses *in utero* and 315 R to kill half of those delivered, within the next 30 days, so that 100 R is far below the LD/50 exposure level; see Rugh and Wohlfromm, 1962, 1965). All three levels of exposure, 5, 50, and 100R, are traumatic at certain earlier stages of development, but have no gross morphological effects on the fetus at 15 days' development.

Exposure at 15 days would hit the developing hematopoietic system at the time of active differentiation and should result in altering the development of that system, at least at the higher level of exposure. To determine whether such alteration could be detected in the peripheral blood, the fetuses were sacrificed at 17 days or 2 days after x-irradiation. This is because in the adult the maximum depression in terms of lymphopenia occurs at about 48 hours after exposure.

Hemoglobin percentage was not seriously altered. Those mice that were allowed to develop to three weeks after birth and then tested showed hemoglobin percentage close to the control average of 14%, with that of the male slightly lower in both the controls and the x-irradiated at this time.

The average white blood cell counts of the controls at 17 days' gestation were slightly higher for the males than for the females. Following x-radiation the males showed a lesser degree of leukocytosis, but the response of all was an increase (leukocytosis) rather than a decrease (leukopenia) in white cell count, which would be expected of the adult. In fact the 100 R female fetal white count at 17 days approached that of the normal 3-week-old controls. The 100 R mice which were allowed to come to term and develop through weaning showed at 3 weeks a white cell count average very close to that of the unirradiated controls. Thus, either the dose of x-rays at 15 days' gestation was sufficient to kill them before maturity (no evidence) or there was complete recovery by this time.

During the two-day post-irradiaton development of the fetus, there was a slight drop in the red cell counts below the average of the parallel controls of both males and females, indicating a mild anemia. For the females the drop was graded according to dose level. Again, by 3 weeks after birth those x-rayed and the controls showed the same average erythrocyte count with only a slight persistent anemia of the males. But during this interval the average level of such x-rayed males had increased from 2,876,000 to 7,086,000 red cells per cubic millimeter, which therefore showed no effect on erythropoiesis.

Finally, all irradiated fetuses showed average depressions in the platelet counts, the most significant of which happened to be the fetal males exposed to the

Table I

	Blood cell counts of mice x-rayed at 15 days' gestation and examined subsequently*							
	Hemoglobin %		White blood cells		Red blood cells		Platelets	
	Males	Females	Males	Females	Males	Females	Males	Females
Control fetuses at 17 days	8.89	8.25	1720	1533	3,438,000	3,244,000	452,333	454,000
15 days + 15 R at 17 days	8.69	7.94	1600	1400	3,204,000	3,326,000	291,000	346,000
15 days + 50 R at 17 days	10.10	10.14	2,360	2,660	3,260,000	3,116,000	385,000	401,000
15 days + 100 R at 17 days	8.09	8.59	3,520	4,740	2,867,000	2,817,000	391,000	363,000
Controls at 3 weeks of age	13.9	14.86	5,140	5,880	7,514,000	7,531,000	1,063,000	1,008,000
15 days + 100 R at 3 weeks	12.82	14.18	5,260	5,420	7,086,000	7,514,000	941,000	1,149,000

* Note: For the controls there were 15 for each average of males or females, but for those x-rayed the averages were of 10.

lowest level. While these data come from the averages of 10 different fetuses, this deviation from the other averages cannot be explained. However, when we examine the males at 3 weeks of age we find even there a persistence in the depression of the platelet counts, even though the figures have risen considerably and are rather close to *those* of the controls.

The data show that counts of the primary blood elements following x-ray exposures of the fetus at 15 days do show some transient alterations, seen at 17 days' gestation, but those mice which survive birth, and live to 3 weeks of post-natal life, appear to have made a full recovery from these blood deviations.

The differential counts were not significantly different for those x-rayed *in utero* and the parallel controls, either at 17 days gestation or at 3 weeks of age. There was a variety of immature cells in both the x-rayed and the controls, but not significantly different in the two groups.

Discussion

A recent study with rat embryos x-rayed at 18 days' gestation to 270 R (Reincke, 1962) showed a response by 8 to 12 days after exposure, and an over-recovery pattern with a peak at 50 days. The surviving rats showed no permanent ill effects or deviations from the normal in the blood elements following this rather high level of exposure at 18 days' gestation. It seems that following x-radiation there may be an early abortive rise in the release of new red cells followed by a true recovery of the hematopoietic system to the normal level (Okunewick and Kretchmar, 1967).

A few studies on the human fetus exposed *in utero* at Nagasaki (Tomonaga, 1962) or Hiroshima (Takamura and Ueda, 1961) and a more recent study of 233 mothers who had had abdominal x-ray examinations during pregnancy did not show any increase in the incidence of leukemia (Wells and Steer, 1961). But other studies have suggested that fetal x-irradiation resulted in incomplete recovery (Baum and Allen, 1959; Krebs *et al.;* 1959; Spalding *et al.;* 1961; Storer, 1961, 1964; Dalrymple *et al.;* 1963). The degree of irreparable damage varied with the level of exposure and seemed to be limited to a maximum of about 8% in the survivors. The test is usually either a reduction in the LD/50/30 or in the shortening of life expectancy. A study of the mouse fetal exposure suggests that the hematopoietic tissue should be included in the tissues that do not fully repair (Christensen and Jackson, 1967).

The embryo and fetus appear to be more radiosensitive than the adults of the same form (Rugh and Wohlfromm, 1962, 1965) or at least they manifest their sensitivity in a graphic form as in congenital anomalies. It is known that the differentiating cell, such as the neuroblast, osteoblast, or erythroblast, is more sensitive than the cell into which it differentiates. Mouse embryonic development is telescoped into a short period of 20 days, but the 15 days' gestation there is active hematopoiesis. One would therefore expect that the blood-forming potentialities of the fetus at this time would be particularly radiosensitive.

The blood forming organs are known to be particularly radiosensitive but there must always be a direct action of the ionizing radiations since there is no evidence of any abscopal effect (Barakina, 1961). Whole blood taken from any peripheral source appears to be rather radio-resistant. Thus, in an embryo or fetus where the organs are forming rapidly and beginning to supply cells for circulation through the blood vascular system one would expect hypersensitivity to ionizing radiations. However, instead of causing immediate leukopenia or delayed leukemia, as might occur in the irradiated adult, exposure of the 15-day fetus causes transient leukocytosis and anemia. But both of these deviations from the normal are rectified very quickly so that as early as 3 weeks after birth one cannot distinguish between those x-rayed *in utero* and the parallel controls, at least with regard to the circulating blood cell types.

It is fortunate that in the fetus this hypersensitive system reacts as it does, in contrast with the way it reacts in the adult, for it appears that complete recovery is achieved shortly after birth. This may reflect the general recuperative powers of the fetus as opposed to the adult. It seems likely that any irreparable damage following fetal irradiation must apply to other and non-vascular systems, especially since 100 R has been shown to cause cataracts (Rugh *et al.,* 1964a) and stunting (Rugh *et al.,* 1964b) in mice x-rayed at 15 days' gestation.

SUMMARY AND CONCLUSIONS

1. In the fetus at 17 days, two days following x-irradiation *in utero* (at 15 days), there were increments in the white cell counts correlated with the level of exposure to x-rays, after both 50 R and 100 R. This is in contrast to the expected reaction of adult mice of the same strain.

2. At 17 days the erythrocyte counts showed a significant depression following 100 R exposure at 15 days' gestation. This too is in contrast with the adult reaction.

3. All platelet counts of those mice x-rayed at 15 days and examined at 17 days' gestation were depressed below the control levels, but by 3 weeks post-natal development they were within the normal range.

4. Both red and white blood cell counts of fetally x-rayed mice returned to normal by 3 weeks after birth.

5. Exposure of the 15-day fetus resulted in a defensive reaction expressed in temporary leukocytosis, as opposed to the normal leukopenia of adult mice similarly exposed, but by 3 weeks of post-natal age both the differential and all other cell counts were within the normal range. This means that effects on the fetal blood (hematopoiesis) are temporary, since there was no evidence of any mortality at the levels of radiation used nor of any permanent effects on the blood counts.

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EXPERIMENTAL EVIDENCE FOR A GONADAL-FAT BODY RELATIONSHIP IN TWO TEIID LIZARDS (AMEIVA FESTIVA, AMEIVA QUADRILINEATA)[1]

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Lipid storage within discrete postcoelomic fat bodies is common among temperate-climate reptiles (Barwick, 1959; Rollinat, 1934; Miller, 1954; Hahn and Tinkle, 1965; Tinkle, 1962; Hoddenbach, 1966). The adaptive significance of the fat body and its cycle has been implied by Tinkle (1962) in the biennial reproductive cycle of the rattlesnake *Crotalus atrox*. Hahn and Tinkle (1965) have experimentally shown that the adaptive significance of an ovarian-fat body relationship in the iguanid lizard *Uta stansburiana* is a rather specific one in terms of support to the vitellization of first clutch follicles which occurs at a time when feeding conditions are below optimum (mid-February in western Texas). Lipid stores in the iguanid *Anolis carolinensis* seem more important in the winter survival of both sexes (Dessauer, 1955). A testicular-fat body relationship has not, however, been demonstrated experimentally though speculation is that lipid stores and their mobilization would have special value during breeding seasons when activity is generally increased. Church (1962) found no fat bodies in his monthly samples of three species of Javanese house geckos. Very few other tropical reptiles have been examined with reference to fat body development.

The present investigation was undertaken to determine the extent of fat body development and its relation to the gonadal cycles in two species of teiid lizards, *Ameiva festiva* and *Ameiva quadrilineata,* from sympatric populations in eastern Costa Rica. Large field samples were examined with regard to gonadal development and fat body size. Ecological information obtained included photoperiod, thermoregulatory behavior, and activity cycles. These data proved valuable in arriving at a possible adaptive significance of a gonadal-fat body interrelationship. Experiments were designed to determine whether lipid mobilization is to some degree regulated by the gonads and whether or not fat bodies are specifically essential to the vitellization of ovarian follicles.

In this report data from the analysis of field samples are presented as well as the results from the experimental phase of the study—ovariectomy, ovariectomy + estradiol-17β replacement therapy, orchidectomy, orchidectomy + testosterone propionate replacement therapy, and fat body excision in females.

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Methods and Materials

Between July 19 and September 6, 1963, 225 specimens of *Ameiva festiva* and 172 of *A. quadrilineata* were collected in banana groves at Pandora, Limon Province, Costa Rica; another 86 specimens of *A. festiva* and 85 of *A. quadrilineata* were examined between July 15 and September 11, 1964. Smaller samples were collected monthly between October 1, 1965, and May 31, 1966. All specimens comprising field samples were killed and examined shortly after they were collected. The total body weight and fat body weights were recorded to the nearest milligram. Body lengths (snout-vent lengths) were recorded to the nearest millimeter. Fat bodies were excised from etherized females through a pair of diagonal ventral incisions about 8 mm. in length above each fat body. Ovariectomies were performed through a single midline incision extending from the 17th to the 20th ventral scute; orchidectomies were performed through a single midline incision extending from the 13th to the 20th ventral scute. Incisions in these operations were closed with 2 to 4 interrupted silk sutures. Lack of ice or refrigeration prevented controlled reduction of the body temperature prior to surgery. Instead, specimens were operated upon only between the hours of 4 AM and 6 AM when body temperatures were lowest, averaging 25 ° C. (body temperatures ranged from about 25 to 39 ° C. during a 24-hour period). All animals employed in these experiments were sexually mature. Female specimens of *A. festiva* over 77 mm. in body length and of *A. quadrilineata* over 64 mm. in body length proved sexually mature as they contained deutoplasmically active follicles. Females with oviducal eggs were not employed. Male specimens of *A. festiva* over 74 mm. and of *A. quadrilineata* over 59 mm. in body length were sexually mature with large testes, noticeable convoluted seminiferous tubules, and hypertrophied epididymides. Lack of any distinct seasonal initiation of vitellization of follicles in females or spermatogenesis in males discouraged the use of juveniles in these experiments since the interval before the onset of such gonadal activities could not be accurately estimated. In sham-operated females the ovaries were exteriorized sufficiently for measuring all yolked follicles present. Field controls were collected and autopsied on days coinciding with autopsy dates of the experimental and sham-operated groups.

Experimental and sham-operated animals were maintained in screen wire cages about 9 $ft.^2$ Each cage contained from 6 to 10 specimens, all of the same sex and species and representing one distinct experimental or sham group. They were provided with constant water and larvae of *Tenebrio molitor* were introduced into the cages several times each day. Cages were kept out of doors and thus exposed to near field conditions of temperature and lighting for July and August in Costa Rica.

Results

Reproductive cycles and fat body size

Deutoplasmic activity in unyolked follicles commences in *A. quadrilineata* with snout-vent lengths greater than 64.0 mm. and in *A. festiva* with snout-vent lengths greater than 77.0 mm. Sexual maturation and egg-laying are not seasonal phenomena and occur during all 12 months of the year. The interval between the initiation of deutoplasmic activity within a set of follicles and ovulation is approximately eight weeks in both species. Oviposition was observed 17 to 21 days after ovulation. The initiation of deutoplasmic activity in another set of follicles does not occur until four to six weeks after oviposition. Field samples for all 12 months of the year yielded a range in fat body size (both fat bodies) in female *A. quadrilineata* of from 0.10 to 2.55% of the total body weight (TBW); the range in *A. festiva* was from 0.10 to 2.39% of the TBW. Females containing eggs within the oviducts had small fat bodies. Fat bodies comprising more than 1% of the TBW were observed in both immature and mature females. Yolked follicles in females with fat bodies comprising over 1% of the TBW ranged from 3.0 to 10.0 mm. in diameter. Pooling all samples of these sexually mature females of both species, the mean fat body percentage of the TBW was 1.7 and the mean follicle diameter was 5.0 mm. Since female *A. festiva* greater than 87.0 mm. in length and *A. quadrilineata* greater than 70.0 mm. in length frequently had fat body percentages much greater than 1% of the TBW, it is apparent that partial depletion and subsequent recrudescence of the lipid content of fat bodies must occur throughout the adult life of the female.

TABLE I

The effect of ovariectomy and ovariectomy + estradiol-17β replacement therapy upon fat body weight in Ameiva quadritineata and Ameiva festiva. Mean per cent fat ± one standard deviation is the percentage of the total body weight comprised by both fat bodies. Per cent fat range is the range of the percentage of total body weight comprised by both fat bodies. Snout-vent (s-v) lengths in millimeters, body weights in grams

Number of animals:	Treatment:	$\bar{x}$ s-v length:	$\bar{x}$ body weight:	% fat range:	$\bar{x}$ % fat:
		Ameiva quadrilineata			
10	ovariectomy	71	8.430	1.33–5.30	3.79 ± 1.40
9	sham operated	73	9.250	0.17–3.02	1.42 ± 1.05
20	control	70	9.350	0.22–2.38	1.06 ± 0.64
6	ovariectomy + estradiol-17β	68	9.528	0.33–0.59	0.47 ± 0.12
		Ameiva festiva			
10	ovariectomy	81	12.176	2.09–4.66	3.40 ± 0.72
7	sham operated	79	12.480	0.60–3.89	1.95 ± 1.54
20	control	82	13.450	0.25–2.40	0.84 ± 0.69
7	ovariectomy + estradiol-17β	81	13.946	0.13–0.59	0.34 ± 0.15

Male specimens of *A. quadrilineata* with snout-vent lengths greater than 59.0 mm. and of *A. festiva* with snout-vent lengths greater than 74.0 mm were found to be sexually mature and in breeding condition regardless of the time of year they were collected. Involution of the seminiferous tubules was not seen in any sexually mature male nor was any seasonal variation in amounts of interstitial cells noted. The percentage of the TBW comprised by both fat bodies in males from field samples ranged from 0.05 to 1.09 in *A. festiva* and from 0.07 to 0.89 in *A. quadrilineata.*

Effect of gonadectomy upon fat body size

Ovariectomy in sexually mature females of both species evoked a significant increase in the mean percentage of the TBW comprised by both fat bodies when compared with percentages in both sham-operated and field control animals (Table I). These females were autopsied 40 days after ovariectomy. There was an increase also in the fat body percentage of the TBW in sham-operated females compared with field controls but this is probably attributed to the constant food supply and with the decreased activity of these lizards in captivity. Sexually mature females of both species were ovariectomized and injected intraperitoneally with 10 μg. of estradiol-17β every two days for a period of 20 days. Mean fat body percentages of the TBW in these animals were much lower than those in any other group of females.

TABLE II

The effect of orchidectomy upon fat body weight in Ameiva quadrilineata and Ameiva festiva and the effect of orchidectomy + testosterone propionate replacement therapy upon fat body weight in A. quadrilineata. Legend same as in Table I

Number of animals:	Treatment:	x̄ s-v length:	x̄ body weight:	% fat range:	x̄ % fat:
	Ameiva quadrilineata				
6	orchidectomy	74	9.958	1.06–2.49	1.87 ± 0.52
23	control	70	10.753	0.10–1.32	0.38 ± 0.27
7	orchidectomy + testosterone	72	10.603	0.06–1.29	0.51 ± 0.43
	Ameiva festiva				
6	orchidectomy	89	19.555	1.20–2.58	1.87 ± 0.46
14	control	86	18.792	0.04–1.45	0.41 ± 0.44

Orchidectomy was performed upon sexually mature males of both species and the animals autopsied 40 days later. The results (Table II) indicate that the percentages of the TBW comprised by both fat bodies were much higher in the castrated animals than in the controls. Sexually mature male *A. quadrilineata* were castrated and injected intraperitoneally with 1.0 mg. of testosterone propionate every two days for a period of 20 days. The mean percentage of the TBW comprised by both fat bodies in this group was nearly 73% less than the mean percentage in the castrated group.

Effect of fat body excision upon follicle development

The importance of nutritional states in relation to the vitellization of follicles in reptiles has not received much attention. Follicular atresia does frequently occur when females are exposed to poor diet in captivity. The occurrence and amount of follicular atresia in field samples has been reported in various lizards and its frequency varies widely from group to group. Follicular atresia was observed in numerous females comprising field samples of *Ameiva*. In the samples examined 12% of the female *A. festiva* and 26% of the *A. quadrilineata* contained at least one atretic follicle. This difference in the frequency of atresia is important statistically in regards to the reproductive potential (mean clutch size) calculated for each species (roughly 2.3 for *A. festiva* and 2.0 for *A. quadrilineata*).

Seven female *A. quadrilineata* and six *A. festiva* underwent bilateral fat body excision and were maintained in captivity for 40 days; all animals were sexually mature with deutoplasmically active follicles at the beginning of the experiment. Upon autopsy it was seen that fat body excision had induced atresia in all (100%) of the deutoplasmically active follicles. Sham-operated females had frequencies of atresia comparable to those given for field samples.

Mention should be made at this time of the behavioral change observed in those females which underwent fat body excision. Compared with all other groups of experimental and sham-operated animals in captivity, their feeding habits can only be described as voracious. Although food was provided several times daily, satiation was not observed in these females. Unlike all the other lizards maintained in captivity these females fed even in early morning and late afternoon when activity was normally suspended. This is interesting since an increase in feeding was not sufficient in inhibiting the atresia of deutoplasmically active follicles which ultimately ensued.

DISCUSSION

The results (Table I) in the four groups of female *Ameiva* show that removal of the ovaries caused an increase in fat body size whereas ovariectomy + treatment with estradiol-17β caused a decrease. Likewise, in males, the percentage of the TBW comprised by both fat bodies was much higher in the castrated animals than in the controls. The mean percentage of the TBW comprised by both fat bodies in the castrated *A. quadrilineata* treated with testosterone propionate was approximately 73% less than that in the group which underwent castration alone. It is therefore assumed that estrogen and testosterone replacement therapy are responsible for the inhibition of an increase in fat body weight such as was observed in the gonadectomized animals. Hahn and Tinkle (1965) found a close correlation between fat body and egg clutch lipid in *Uta stansburiana*. They also observed a high incidence of follicular atresia in females which had undergone bilateral fat body excision. Hahn (unpublished data) noted an increase in liver lipid due to estradiol-17β treatment. The results from the gonadectomized-steroid treated *Ameiva* strongly suggest a regulatory effect of these sex hormones upon lipid mobilization within the fat bodies. The dosage of estradiol-17β employed was arbitrarily chosen but was probably sufficient in evoking a maximal response within the fat bodies. The dosage of testosterone propionate was assuredly high; histological sections of kidneys taken from these males at autopsy showed sexual segment epithelia hypertrophied beyond the condition seen in normal breeding males, and there was also a much greater than normal abundance of secretory granules present.

Although no measurable correlation was found in sexually mature females between fat body weight and size of yolked follicles (both diameter and weight) this should not imply a lack of relationship between the two structures. Data at hand do indicate that fat body size is always small in females with oviducal eggs as well as in females with ovarian follicles of ovulatory size. The length of time required for the complete vitellization of a set of follicles (about eight weeks) coupled with the possibility that fat body lipid might not be utilized throughout vitellization add to the difficulty in demonstrating close correlation between lipid depletion in fat bodies and yolk increase in ovarian follicles.

Both species of *Ameiva* are heliophilic and are active generally when sunlight is present. During periods of activity thermoregulatory behavior largely involves heat absorption from soil surfaces in areas directly exposed to the sun and heat radiation to soil surfaces in shaded areas. Average monthly air temperatures from field stations within the banana groves at Pandora do not vary significantly and there are no distinct seasons when lizards are completely inactive. There are periods, however, when normal activity is completely suspended and *Ameiva* remain within their burrows. Continuous rainfall typifies such periods of inactivity. Following one rainy period of about eight days in July of 1966 there was no observed reduction in the number of lizards present in the banana groves nor in their reproductive capacity, *i.e.,* there was not an increase in the frequency of follicular atresia in the field samples analyzed. During such periods of inactivity, when feeding is suspended, the adaptive significance of fat reserves for general survival is obvious. The reliance upon such fat reserves for the vitellization of deutoplasmically active follicles in mature females may provide insurance against follicular atresia which might otherwise occur were these reserves not present. Results from the experiments involving fat body excision support this contention. On a population level the presence of sufficient fat reserves in at least some females with yolking follicles during such periods of inactivity would provide for the observed presence of hatchlings during all twelve months of the year.

A specific adaptive significance of lipid reserves in fat bodies is not apparent in males. However, since an androgen-fat body relationship was suggested by the experimental results presented above, it seems reasonable to again speculate that fat reserves may be specifically important in relation to the energy demands imposed during active breeding periods at which time androgen secretion is probably at its height.

Summary

1. Percentages of the total body weight comprised by both fat bodies ranged in field samples of female *Ameiva quadrilineata* from 0.10 to 2.55 and in female *A. festiva* from 0.10 to 2.39. Percentages from field sample males ranged lower, from 0.07 to 0.89 in *A. quadrilineata* and from 0.05 to 1.09 in *A. festiva.*

2. Ovariectomy in both species was followed by an increase in fat body weight compared with field controls and with sham-operated animals. Treatment involv-

ing the administration of estrogen in both species subsequent to ovariectomy inhibited any such increase in fat body weight.

3. Orchidectomy in both species was followed by an increase in fat body weight. Specimens of *Ameiva quadrilineata* which were castrated and also treated with testosterone showed no such increase.

4. Fat body excision in females of both species (sexually mature animals) resulted in the atresia of all deutoplasmically active follicles.

5. An adaptive significance of fat bodies in *A. festiva* and *A. quadrilineata* is suggested in terms of material support to the vitellization of ovarian follicles especially during infrequent periods of inactivity.

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THE ASEXUAL GENERATIONS, LIFE-CYCLE, AND SYSTEMATIC RELATIONS OF MICROPHALLUS LIMULI STUNKARD, 1951 (TREMATODA: DIGENEA)[1]

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Stunkard (1951) described intense and massive infections by encysted metacercariae in the horseshoe crab, *Limulus polyphemus*. The cysts were dispersed throughout the body, even in interommatidial spaces. They were oval, thick-walled, and contained active metacercariae, so well developed that the ovary, testes and vitellaria were almost fully mature and the seminal vesicle contained motile spermatozoa. The cysts were fed to chicks, white mice and golden hamsters. The chicks did not become infected but mice and hamsters yielded sexually mature worms 2 to 4 days after ingestion of the metacercariae. The worms were eliminated rapidly and none were found after 9 days. No mature worms were recovered from subsequent feedings to mice or hamsters, denoting a possible immunity from the initial infection. The rapid elimination of the parasites and subsequent resistance to reinfection indicated that mice and hamsters are not natural or normal hosts of the trematode. Stunkard noted that the worms were similar to *Microphallus claviformis* (Brandes, 1888), a parasite of shore-birds in Europe, and predicated (p. 315), "It is entirely possible that the metacercariae in *Limulus* may mature in shore-birds. When the asexual stages and cercariae are discovered, it will be possible to attempt infection of crustaceans and learn whether or not the parasite can use both arachnids and crustaceans as second intermediate hosts. Since microphallid cercariae are very immature and the metacercariae undergo extensive development in the second intermediate host, it must be more than a mere transfer host." The following year, laboratory-reared birds became available and Stunkard (1953) reported that herring gulls, *Larus argentatus,* can serve as natural hosts of *M. limuli.* At that time the first intermediate host and asexual generations were yet unknown. During the summer of 1967, the larval stages were discovered and experimental infection of *Limulus polyphemus* was accomplished.

The genus *Microphallus* has been reviewed repeatedly but its limits and valid species are still uncertain. It was erected by Ward (1901) to contain *Distomum opacum* Ward, 1894, a parasite of the intestine of the bowfin, *Amia calva,* taken in Lake St. Clair, Michigan. Over 1000 worms were found in a single fish. The stomach contained many (26) partially digested crayfish, *Cambarus propinquus.* The cephalothorax of the crayfish, above the heart and gonads, contained encysted metacercariae, specifically identical with excysted juvenile and sexually mature specimens of *D. opacum* present in the intestine. The parasite was found in small

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numbers in the channel-cat, *Ictalurus punctatus,* and the yellow perch, *Perca flavescens.* A second species, *Microphallus ovatus,* was described by Osborn (1919) from cysts in the liver of unidentified crayfish and the intestine of small-mouth black bass, *Micropterus dolomieu,* taken from Lake Chautauqua, New York. The parasite was reported also from the bullhead, presumably *Ameiurus nebulosus* (LeSueur). Van Cleave and Mueller (1934) reported *M. opacus,* from *Ameiurus nebulosus* and *Ambloplites rupestris,* and *M. ovatus* from *Esox niger* and *A. rupestris,* taken in Oneida Lake, New York. They stated (p. 29), "The resemblance of *M. opacus* and *M. ovatus* is rather close. Moreover, what differences exist are largely bridged by variations. It seems not impossible that a comprehensive study of these forms might show them to be different modifications of the same species induced by the host relationship or other factors. In fact, a few of our specimens possess such clearly intermediate characteristics that positive determination was impossible." At that time, all specimens of *Microphallus* had been described from fishes. Rausch (1946a) reported heavy infections by *M. ovatus* in three species of turtles, *viz., Chrysemys belli marginata, Chelydra serpentina,* and *Graptemys geographica.* Later, Rausch (1946b), from a raccoon, *Procyon lotor,* reported specimens that belonged to the genus *Microphallus* but differed from both *M. opacus* and *M. ovatus.* He declared (p. 209), "Evidence is at present insufficient to justify describing the specimens from the raccoon as new, since it is impossible to determine whether the differences are specific, or have been brought about by development in a unusual host animal. It would be very desirable to attempt infection of different animals with metacercariae of *Microphallus ovatus* in order to gain more information concerning host specificity in this group." Results of such attempts were reported by Rausch (1947). Metacercariae from *Cambarus propinqus* were employed. He found that the cysts fell into two distinct size groups, the larger of which (1.3 by 0.9 mm.) agreed with the measurements given by Ward (1894) for cysts of *M. opacus.* Although the cysts were of two sizes, even from the same crayfish, the metacercariae were reported to be morphologically identical and all were able to mature when fed to suitable experimental hosts. He was unable to excyst the larvae by mechanical means, but found that they emerged in 48 hours when placed in Ringer's solution with fragments of the gland in which they were found. On release, they began to copulate and when left in normal saline, Ringer's solution, or media devised for the culture of Protozoa, they matured and produced large numbers of apparently normal eggs. He fed metacercariae to various kinds of possible hosts; the cysts failed to hatch in chicks and toads; the metacercariae emerged but were voided in an immature condition by certain snakes. Sexually mature worms were recovered from two species of turtles, two snakes, the opossum and the raccoon, but only negative results were obtained with salamanders, frogs, chicks, albino rats and a skunk. Adults from the two types of cysts differed in size, but since they otherwise were so similar, Rausch concluded that they belong to a single species, *M. opacus.* He noted that egg-production is much more abundant in reptilian and mammalian hosts, but infections are of much longer duration in reptiles and fish. Accordingly, he suggested that cold-blooded animals are the normal hosts, and that others are accidental hosts in which rapid and copious egg-development occurs before the trematodes are eliminated. The first intermediate hosts, larval stages, and source of infection of crayfish were unknown, so means for experimental control of host-specificity were not available. Indeed, they have not yet been identified.

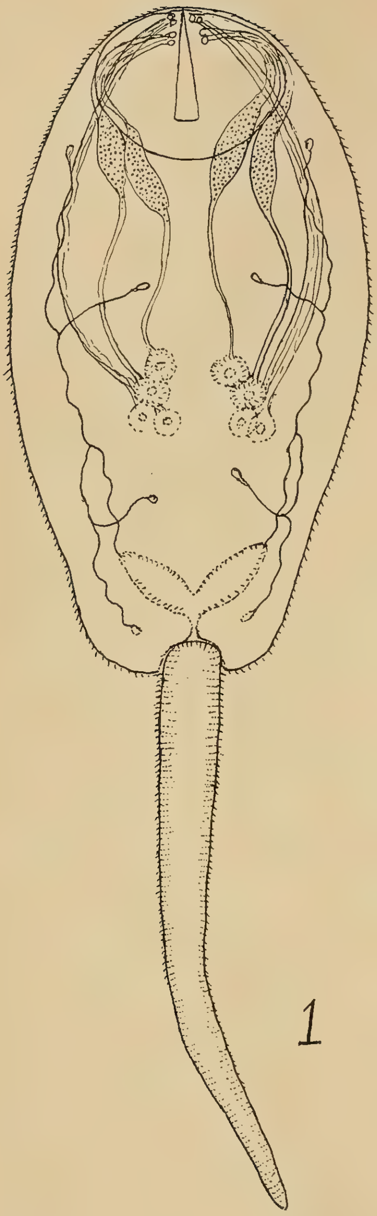
The first clues to the complete life-cycle of microphallid trematodes were provided by Marie Lebour. She (1912) redescribed *Cercaria ubiquita* Lebour, 1907 from *Littorina obtusata* and *Littorina rudis* (= *L. saxatilis*) as the larval stage of *Spelotema excellens* Nicoll, 1907. Actually, *C. ubiquita* Lebour (1907) was described from *Paludestrina stagnalis* and Deblock and Rosé (1966, p. 300) recognized it as the larval stage of *Maritrema subdolum* Jägerskiöld, 1909 as described by Deblock, Capron and Rosé (1961) and quite distinct from *C. ubiquita* Lebour, 1912. According to Deblock and Rosé (1966), *Paludestrina stagnalis* is a synonym of *Hydrobia acuta*. Lebour (1912) reported that the cercariae from the species of *Littorina* bored into the tissues of the crabs, *Carcinus maenas* and *Cancer pagurus,* and developed into "the ordinary *Spelotrema* form." She compared excysted metacercariae with specimens of *Spelotrema excellens* Nicoll, 1907 and declared (p. 433), "there can be little doubt that *Cercaria ubiquita* is the young form of *S. excellens* the first host thus being *Paludestrina stagnalis, Littorina obtusata* and *L. rudis.* The intermediate host *Carcinus maenas* and *Cancer pagurus* and the final host probably the herring gull *Larus argentatus.*"

The first report of an experimentally demonstrated life-cycle in the family Microphallidae, that of *Cornucopula nassicola* (Cable and Hunninen, 1938) Rankin, 1939, was made by Rankin (1939b) in an abstract published in December. But another and more complete account was already in press and Cable and Hunninen (1940) gave the first detailed description of the successive stages of a microphallid trematode, *Spelotrema nicolli* n. sp. The asexual stages were in the snail, *Bittium alternatum,* the metacercariae in the blue crab, *Callinectes sapidus,* and adults were obtained from feeding the metacercariae to gulls, *Larus argentatus.* As noted by Cable and Hunninen, the generic name, *Spelotrema* Jägerskiöld, 1901, is a subjective synonym of *Levinseniella* Stiles and Hassall in Ward, 1901. Rankin (1940) amplified his (1939b) abstract; he reported that *Cercaria nassicola* Cable and Hunninen, 1938 encysts in the pericardial sinus of the sand-flea, *Talorchestia longicornis,* and develops in gulls, *L. argentatus,* into adults specifically identical with worms that he (1939a) had described from natural infections of piping-plovers, *Charadrius melodus* Ord., and spotted sandpipers, *Actitis macularia* (Linn.) as *Cornucopula sippiwissetensis* n.g., n. sp. Meanwhile, Yamaguti (1939) had erected the genus *Gynaecotyla* to contain a species with two ventral suckers that he (1934) had described as *Levinseniella squatarolae.* Rankin (1939a) had included *L. squatarolae* in his genus *Cornucopula,* but since the paper by Yamaguti appeared first, *Cornucopula* became a synonym of *Gynaecotyla.* Rankin (1940) recognized the synonymy and listed the species as *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939. But Yamaguti did not include the species, *nassicola,* in *Gynaecotyla;* the new combination was made by Rankin and the correct name of the worm is *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Rankin, 1940. Baer (1943) suppressed *Spelotrema* as a synonym of *Microphallus* and transferred all the species from *Spelotrema* to *Microphallus.*

According to Deblock and Rosé (1966), the life cycle of *Microphallus* *pygmaeus* (Levinsen, 1881) was reported by Belopolskaia in 1949, but no publication of that date was cited. Apparently the data were taken from Belopolskaia (1952). These authors stated that the parasite occurs in *Littorina rudis* and *L. obtusata;* the cercariae lack tails, stylets and glands of penetration; they complete their development in their sporocysts, with no free aquatic stage. The account is supported by the findings of Ching (1961) and (1962). She reported sporocysts in *Littorina scutulata* that contained metacercariae which, when fed to mice, developed into gravid *M. pygmaeum.*

The next complete life-cycle was elucidated by Stunkard (1957). He confirmed, in part, the postulate of Lebour (1912); showed that *Cercaria ubiquita* of Lebour (1912) from *Littorina obtusata, L. saxatilis,* and *L. littorea* taken at Woods Hole, Massachusetts, is identical with the metacercaria in *Carcinides maenas* described by M'Intosh (1865) and is the larval stage of *Microphallus similis* (Jägerskiöld, 1900) Baer, 1943. Schiller (1959) purported to describe the life-cycle of *Microphallus pirum* (Afanassiev, 1941) Rausch, 1953. But as noted by Cable (1963), Schiller's experimental procedures were faulty; he confused two types of cercariae as the "early stage" and "completely developed" larva of a microphallid parasite in Alaska. The first type is a microphallid, whereas the second is a plagiorchid species, similar to the cercaria of *Renicola thaidus* as described by Stunkard (1964). Ching (1965) stated that Schiller's account is incorrect, but she rejected the suggestion of Biguet, Deblock and Capron (1958) that *Microphallus enhydrae* Rausch and Locker, 1951, is identical with *M. pygmaeum.* Deblock and Rosé (1966) noted discrepancies in the report by Schiller: the failure to obtain infection of crustaceans and the improbability that the large, pharyngeate cercaria depicted in Figure 4, was actually a microphallid species. They concluded that the mollusks utilized in the experiments harbored previous natural infections. Deblock, Capron and Rosé (1961) called attention to a similar error by Hunter and Vernberg (1953) in the reported life-cycle of the microphallid species identified as *Gynaecotyla nassicola* (Cable and Hunninen, 1938). Strangely, the error by Hunter and Vernberg was an exact repetition of the one made by Glading (1935).

Deblock and Rosé (1964) described a microphallid species from wild ducks, *Anas platyrhynchos* L., taken along the Somme near Boves, France, as *Atriophallophorus samarae* n. g., n. sp., and suggested that *Levinseniella minuta* Price, 1934 may belong in the new genus. The latter species was described from the intestine of the scaup duck, *Nyroca affinis,* taken at Flamingo Lake, Culebra Island in the West Indies. Stunkard (1958) described the morphology and life-history of the species from material taken in New England, U. S A.. He found that there are at least two generations of sporocysts, that the cercariae emerge from the sporocysts in a relatively immature condition, without tails, stylets or penetration glands, and that they encyst in their molluscan hosts, *Hydrobia minuta* and *Amnicola limosa.* Since the miracidia emerge only after ingestion by the snail-host, there is no free-swimming stage in the life-cycle. The metacercariae developed in their cysts and matured in white mice and golden hamsters. Worms from smaller and younger cysts required longer to reach maturity in the final host and become gravid at a slightly smaller size. Eggs from worms matured in a mouse were fed to *H. minuta* and all of ten specimens exposed



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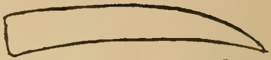

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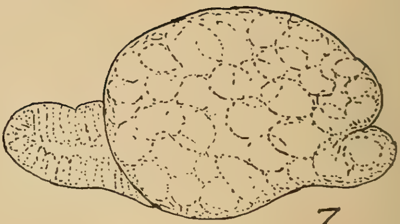

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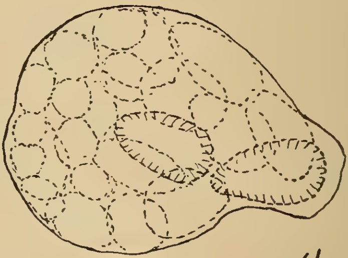

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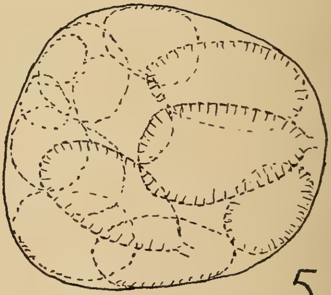

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Figures 1–5.

Figure 1. Cercaria, drawn from pencil sketches of living larvae to show the morphology of the larva.

Figure 2. Stylet, lateral view, from living larva.

became infected. The account of Stunkard was confirmed by Burns (1963) who found the parasite in the snail, *Oxytrema silicula* (Gould), on the West Coast of the United States. Adults were found in natural infections of domestic ducks and developed experimentally in hamsters. After examination of the type-specimens of *Levinseniella minuta* Price, 1934 and others provided by Burns, Deblock and Rosé (1965: 429) announced the identity of "*A. samarae* Deblock et Rosé et de *L. minuta sensu* Stunkard, 1958. It est donc vraisemblable que le nom correct de l'espèce puisse définitement être *Atriophallophorus minutus* (Price, 1934) Deblock et Rosé, 1964 n. comb." The authors also redescribed *Levinseniella somateriae* Kulatschkowa, 1958, from *Anas platyrhynchos* taken in France. The species had been described from eider ducks taken at Kandalakschska, on the White Sea, and Deblock and Rosé (1962) had questioned its position in the genus *Levinseniella;* after study of specimens taken from ducks in France, they announced the transfer of the species to *Microphallus* as *M. somateriae* (Kulatschkhowa, 1958) n. comb.

The validity of the species described by Stunkard (1951) as *Microphallus limuli* has been questioned. Stunkard reported the morphological similarity between *M. limuli* and *M. claviformis* and distinguished between the two species on largely bionomic features. Biguet, Deblock and Capron (1958) made a comprehensive review of the family Microphallidae. They accepted the action of Baer (1943) who suppressed *Spelotrema* Jägerskiöld, 1901 and *Monocaecum* Stafford, 1903 as synonyms of *Microphallus* and of Stunkard (1951) who added *Spelophallus* Jägerskiöld, 1908 to the list of synonyms. Biguet, Deblock and Capron emended the generic diagnosis of *Microphallus* to contain the species from these genera and those of *Carneophallus* Cable and Kuns, 1951. They gave a list of accepted species with a key to diagnostic characters, hosts, and geographic distribution. Concerning *M. limuli,* they noted that the species was based in part on bionomic characters and predicated (p. 426), "Le peu de spécificité parasitaire des *Microphallus* ne nous paraît pas justifier ce point de vue, surtout à propos d'un second hôte intermédiare que, par ailleurs, a une biologie côtière et se nourrit surtout de Mollusques (L. Fage, *in* P. Grassé, 1949, VI, p. 219). Nous proposons donc que *M. limuli* tombe en synonymie devant *M. claviformis.*"

Although *M. claviformis* was known from several descriptions, the early stages remained indefinite. Deblock (1960) expressed the belief that metacercariae from the amphipods, *Corophium grossipes* and *Gammarus duebeni,* described by Lebour (1908) as *Cercaria corophii,* are larvae of *M. claviformis.* Reimer (1963) identified metacercariae from amphipods and isopods taken in the Baltic as juvenile stages of *M. claviformis.* Rebecq (1964) repeated, confirmed and amplified the account of Deblock, Capron and Rosé (1961) on the life-cycle of *Maritrema subdolum;* and gave a detailed description of the stages in the life-cycle of *Microphallus papillorobustus* (Rankin, 1940) Baer, 1943 (syn. *M. chabaudi* Capron, Deblock et Biguet, 1957). Also, he reported that meta-

FIGURE 3. Sporocyst, natural infection, fixed and stained specimen, 0.06 mm. long, with germ balls and tip of an attached lobe of the digestive gland of the snail.

FIGURE 4. Sporocyst, natural infection, fixed and stained specimen, 0.100 by 0.070 mm., with germ balls and developing cercariae.

FIGURE 5. Sporocyst, natural infection, fixed and stained specimen, 0.120 by 0.110 mm., with fully-formed cercariae.

cercariae from the crustaceans *Sphaeroma hoockeri, Idotea baltica* and *Orchestia gammarella* became sexually mature *M. claviformis* in 24 hours when fed to ducklings and in 30 to 35 hours when placed in an incubator at 40 ° C. but failed to develop in gulls, *Larus argentatus.* Other metacercariae from *Gammarus locusta* and *Idotea baltica,* which matured in the incubator at 39–40 ° C, and in domestic ducklings and *L. argentatus,* were described as *Microphallus hoffmani* n. sp., morphologically similar to *M. excellens* (Nicoll, 1907). Other metacercariae from the same hosts, matured in the incubator at 40° and in *L. argentatus,* but not in domestic ducklings; they were identified as *Levinseniella tridigitata,* a species described by Deblock, Capron and Biguet (1958) from the shore-birds, *Actitis hypoleucos* (L.) and *Charadrius hiaticula* (L.). Rebecq also described a microcercous cercaria, produced in sporocysts in *Hydrobia ventrosa,* which he designated as *Cercaria camarguensis;* it was assigned provisionally to the Microphallidae without generic designation.

Deblock and Rosé (1966) listed five species of the genus *Microphallus* for which the life-cycles had been experimentally demonstrated, *viz., M. nicolli* (Cable and Hunninen, 1940) by the authors; *M. pygmaeum* (Levinsen, 1881) by Belopolskaia in 1949; *M. similis* (Jägerskiöld, 1900) by Stunkard in 1957; *M. somateriae* (Kulatschkowa) by Tchoubryk in 1957 and Kulatschkowa in 1958, as reported by Belopolskaia (1963); and *M. papillorobustus* (Rankin, 1940) by Rebecq in 1964. Other accounts of life-cycles were questioned on grounds of (1) difficulty of specific determination, (2) errors of interpretation, and (3) lack of experimental confirmation. These authors described cercariae from natural infections of *Hydrobia acuta* (syn. *Paludestrina stagnalis*) which penetrated and developed into metacercariae in isopods and amphipods, *Sphaeroma serratum* and *Orchestia gammarella.* The metacercariae, excysted mechanically or in the digestive tract of a white mouse, were identified as *Microphallus claviformis* (Brandes, 1888), identical with specimens found by Deblock and Rosé (1965) in the intestine of *Nyroca ferina* (L.) taken in the Boves area of the Somme. Infection of snails was not accomplished but the authors stated (p. 310), "Il ne paraît pas que les trématodes décrits puissent être attribués à une autre espèce que *M. claviformis,* fréquemment rencontrée chez de nombreux hôtes définitifs de toute l'Europe occidentale ou septentrionale. En ne tenant compte que de la taille de la papille mâle, il ne pourrait lui être opposé que trois autres especès: *M. limuli* Stunkard, 1951 et *M. nicolli* Cable et coll., 1940 en Amérique du Nord; *M. longicolle* Yamaguti 1939 au Japon; mais toutes ces espèces s'excluent pour des raisons morphologiques ou biologiques; la taille de leur corps est voisine ou supérieure à 400 μ, leur second hôte intermédiaire n'est pas un Isopode, mais un Xiphosure ou un Décapode suivant le cas. Le taille de *claviformis* est de 200 à 300 μ; et morphologiquement différente de celle de *nicolli,* sa cercaire est spécifique des Isopodes et des Amphipodes." In a footnote, the authors state that when exposed to cercariae of *M. claviformis,* the decapods, *Carcinus maenas,* do not become infected.

This statement by Deblock and Rosé (1966) implies tacit acceptance of *M. limuli* as a valid species, distinct from *M. claviformis.* Their decision may have been influenced by the failure by Rebecq (1964) to infect herring gulls, *L. argentatus,* by feeding metacercariae of *M. claviformis.*

DESCRIPTIONS

Sporocyst

There are at least two generations of sporocysts. Since experimental infection of snails was not accomplished, primary sporocysts could not be positively identified, but the consistent presence of scores of mature sporocysts containing cercariae, together with numerous very small daughters, is good evidence for more than one generation of sporocysts. The sporocysts (Figs. 3, 4, 5) are very numerous, hundreds in a snail; they occur in clusters and masses in the haemolymphatic sinuses of the digestive gland of the snail. They are spherical to oval, the largest about 0.12 mm. in diameter or, if extended, some 0.20 by 0.065 mm. The wall is thin and delicate with circular and longitudinal muscle fibers below the cuticula. There is a birth-pore, typically at an end, and the cercariae emerge tail foremost. Small daughters, 0.020 mm. in diameter, are filled with germinal cells and incipient germ-balls, all of which stain heavily. Sporocysts 0.06 mm. (Fig. 3) in diameter have well formed germ-balls and later the larvae develop muscular body-walls and become active. At this stage, it is impossible to determine whether the larva is a daughter sporocyst or a cercarial embryo. Small sporocysts can migrate slowly by the initial protrusion of a foot-like projection and subsequent forward progression. Movement of older, gravid sporocysts (Figs. 4, 5) results, in large part, from the activity of the contained progeny.

Cercaria

It has long been known that *Hydrobia minuta* Totten in the New England area harbors two species of microphallid cercariae. The snails were identified by Dr. William J. Clench and grateful acknowledgment is hereby rendered. The two kinds of cercariae are the same size but the species can be distinguished by differences in swimming behavior and by differences in the shapes of their stylets. One of these cercariae attacked and penetrated small horseshoe crabs and proved to be the larva of *Microphallus limuli.* These cercariae also entered and encysted in the isopod, *Erichsonella attenuata,* but natural infections of *E. attenuata* were not observed. It is accepted that normal procedures may be modified under captive conditions. When exposed in the confines of a finger-bowl to hundreds of swimming cercariae, infections may occur that would not happen in natural environments. Stunkard (1953) reported that "All of *L. polyphemus* with a carapace width of more than 3 mm. from the Orleans area were infected." That statement is erroneous; the figure intended was 30 mm. In the present experiments, most of the crabs with a width of 20 mm. were infected. The horseshoe crab does not have a pelagic stage; it is apparent that the young individuals become infected shortly after they reach the mud-flats and undergo the first molt, when about six weeks of age (Shuster, 1953). It is possible to superimpose infections; small crabs were placed in fingerbowls with snails that were shedding cercariae and specimens were dissected at intervals of two days to two weeks. All stages from recently entered, unencysted larvae to older encysted ones were recovered. Finally, eggs of *L. polyphemus* were hatched in the laboratory, and larvae with a capapace-width of 3 to 4 mm. were exposed.

They became heavily infected and the metacercariae from the experimental infections developed in mice to typical *M. limuli.*

The cercariae of *M. limuli* (Fig. 1) swim actively at all levels in the water, the body bent ventrally and contracted until it is almost circular, while the tail, which is subterminal in insertion, is extended and lashes violently in a sculling movement, causing the larva to rise in the water. If the tail is extended the body is contracted, and *vice versa.* During periods of rest, the larva slowly sinks in the water. Under a coverglass, the beating of the tail causes the anterior end of the body to oscillate from side to side, while the posterior end is relatively motionless.

Normally emerged cercariae are oval, flattened dorsoventrally, 0.060 to 0.125 mm. in length and 0.025 to 0.050 mm. in width. When elongated, the sides are almost parallel; when contracted the body may be ovate, wider in either the anterior or posterior portion. The tail is almost round in cross-section; it may extend to a length of 0.125 mm. and is 0.004 to 0.010 mm. wide at the base. The cuticula of the body bears small, scale-like spines, arranged in an imbricate pattern and the tail has some 100 annulations. The stylet (Fig. 2) is simple, an elongate, narrow wedge that turns slightly ventrad toward the tip. It is 0.018 to 0.020 mm. in length. The tip is formed first and material is added at the base as it elongates. The oral sucker is 0.022 to 0.026 mm. in diameter and is the only part of the digestive system now recognizable. There are the usual four pairs of penetration glands, situated in the posterior part of the middle third of the body. The ducts of the two anterior pairs color strongly with vital dyes, neutral red and Nile blue sulphate, displaying brightly colored granules in a pale fluid. The secretion accumulates in the bends just posterior to the oral sucker, but is extruded before encystment. On either side, the duct from the most anterior cell passes forward singly while the duct from the other anterior cell bends laterad and with the ducts from the posterior cells forms a bundle which courses forward above or just medial to the excretory duct. As it nears the oral sucker, the duct from the anterior cell turns laterad above the sucker and the ducts from the two anterior cells become contiguous at the side of the sucker. They pass around the sucker and open near the midline on the ventral surface, near the anterior end of the body but posterior to the tip of the stylet. The posterior gland cells and their ducts do not take stain; their ducts pass around the oral sucker ventral to those of the anterior cells and open near the tip of the stylet. Their function is still unknown. The excretory bladder is V-shaped with a collecting duct passing forward on each side (Fig. 1). Immediately posterior to the penetration glands the duct divides into anterior and posterior branches, each of which divides again to give the formula $2[(1+1)+(1+1)]$. During the metacercarial stage the number of flame-cells doubles to give the pattern reported by Stunkard (1951, Fig. 2).

DISCUSSION

The description and figure by Cable, Connor and Balling (1960) of *M. claviformis* from plovers and an unidentified sandpiper taken in Puerto Rico and the discovery of the larval stages of the species by Deblock and Rosé (1966) permit an objective comparison with corresponding stages in the life-cycle of *M. limuli* as reported by Stunkard (1951) and in the present paper. In addition to bionomic differences, there are distinctive morphological features. The figure of a metacercaria of *M. claviformis* given by Rebecq (1964) shows an immature specimen whereas the one by Deblock and Rosé (1966) depicts a mature metacercaria, and both show differences from the metacercaria of *M. limuli* as represented by Stunkard (1951). The same differences appear in comparison of adults of *M. claviformis* figured by Cable *et al.* (1960) and of *M. limuli* by Stunkard (1951). The metacercarial cysts of *M. limuli* from *L. polyphemus* are slightly larger than those of *M. claviformis* as described by Rebecq (1964). The adults of *M. limuli* are distinctly larger, especially those from birds reported by Stunkard (1953). The worms mature at a smaller size in mammals. In *M. claviformis* the suckers are about the same size, whereas in *M. limuli*, the acetabulum is distinctly smaller than the oral sucker. Furthermore, in *M. limuli* the acetabulum and gonads are situated farther posteriad in the body.

SUMMARY

Cercariae from *Hydrobia minuta* Totten, taken in the Massachusetts area, have been identified as the larvae of *Microphallus limuli* Stunkard, 1951. The sporocysts and cercariae are described; young *Limulus polyphemus* were infected; and adults were recovered after feeding the metacercariae to mice. Systematic relations of species in the genus *Microphallus* are reviewed.

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ECDYSONE AND ECDYSONE-ANALOGUES: THEIR ASSAY AND ACTION ON DIAPAUSING PUPAE OF THE CYNTHIA SILKWORM[1]

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Certain weeds, ferns, and evergreen trees have been found to contain complicated sterols closely resembling or even identical to ecdysone, the growth hormone of insects. Thus far, a total of eight "phytoecdysones" have been extracted, chemically characterized, and shown to be highly active in the puparium assay for ecdysone (for reviews and chemical formulae see Kaplanis *et al.,* 1967; Ohtaki *et al.,* 1967; Staal, 1967; Takemoto *et al.,* 1967).

The endocrine activities of five of the materials have been studied in further detail by Kobayashi *et al.* (1967a, 1967b) in assays performed on brainless pupae of the cynthia silkworm. Pupae injected with 1 μg. are reported to undergo the typical ecdysone response by transforming into brainless but otherwise normal moths. By contrast, the higher dose of 10 μg. caused abnormal development including the formation of what are described as "second pupae and pupal-adult mixtures." As the Japanese investigators point out, this result is incomprehensible since the preservation of pupal characters is the assay, not for ecdysone, but for juvenile hormone.

In the experiments reported here the phenomenon has been subjected to detailed reexamination. Graded doses of six phytoecdysones have been compared with synthetic α-ecdysone in terms of their ability to provoke normal or abnormal development of diapausing pupae of the cynthia silkworm.

MATERIALS AND METHODS

1. *Experimental animals*

Cocoons of *Samia cynthia* were obtained from dealers and stored at 8° C. until used. In control experiments, the chilled pupae were removed from cocoons and placed at 25° C. in incubators programmed for either 12 or 16 hours of daily illumination; all individuals initiated development after 30–50 days and emerged as moths after an additional 19–21 days. Certain experiments were carried out on unchilled cynthia pupae that were stored at 25° C.

2. *Materials assayed*

Synthetic α-ecdysone was provided by Drs. John Fried and John Siddall of the Syntex Research Laboratory. Inokosterone, β-ecdysone, and cyasterone were supplied by Prof. T. Takemoto of Tohoku University. Ponasterones A, B, and

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C were obtained from Prof. K. Nakanishi of Tohoku University. All materials except ponasterone B were crystalline. A weighed amount of each substance was dissolved in 1 part warm isopropanol and diluted with 9 parts water. Two-fold serial dilutions were then prepared in 10% isopropanol.

3. *The biological assay*

Groups of chilled pupae were returned to room temperature and removed from their cocoons. Male individuals weighing approximately 2 g. were selected for the assay. The pupae were anesthetized with carbon dioxide and their brains removed (Williams, 1959). By this maneuver the pupae were stabilized in permanent diapause and could be stored indefinitely at 25° C. without further development. The brainless pupae were kept at room temperature for one day and then used in the assay. Parallel experiments were carried out on chilled pupae from which the brains were not removed; as noted above, uninjected pupae of this type required 30–50 days at 25° C. before initiating adult development. Unchilled cynthia pupae were used in certain other experiments; this material has the advantage of persisting indefinitely in diapause if not injected with ecdysone. All seven ecdysones were also tested on a series of isolated pupal abdomens which were prepared as previously described (Williams, 1959).

TABLE I

Time-table for the adult development of Samia cynthia at 25° C., with special reference to externally visible characters

Day	Developmental events
0–1	The epidermis detaches and begins to retract from the overlying pupal cuticle of the wings; by the 16th hour the tips of the legs show retraction.
1–2	Full retraction of the wing epidermis; leg retraction extends a short distance above the tips.
2–3	The legs show generalized retraction; the forelegs have bulbous tips and show earliest trace of segmentation.
3–4	Forelegs show ovoid tips and clear-cut pigmentation; eye lobes fully formed.
4–5	Legs with elongate segments; the forelegs have white triangular tips.
5–6	[No good characters.]
6–7	Formation of unpigmented facets of compound eyes.
7–8	Eyes show pink pigment.
8–9	Eyes reddish brown.
10–11	Tarsal claws pigmented.
11–12	[No good characters.]
12–13	Barbs of antennae pigmented.
13–14	[No good characters.]
15–16	Pigment appears at base of forewings.
16–17	Forewings show complete pigmentation; abdomen and legs unpigmented.
17–18	Full pigmentation of abdomen and legs; initiation of resorption of molting fluid.
18–19	Full resorption of molting fluid.
19–20	Elongation and distension of body; moth emerges and expands wings.

For the purposes of the assay, groups of pupae were anesthetized with carbon dioxide and 50 μl. of graded doses of each material were injected into the thoracic tergum. A 27-gauge hypodermic needle and a 1-ml. syringe were utilized; the latter was controlled by an Agla micrometer. The puncture was sealed with melted wax and the injected pupae placed at 25° C. under 16 hours of daily illumination.

4. *Evaluation of assays*

Each day the injected animals were examined under a dissecting microscope, the cuticle being locally moistened with 70% ethanol. To eliminate surface reflections, the incident white light was polarized and a crossed polarizing filter was placed just below the objective lens. This maneuver enables one to see inside the cuticle of the intact insect and to follow the day-to-day progress of development.

The onset of adult development could be recognized within 4 to 6 hours in terms of the initiation of the detachment and retraction of the wing epidermis from the overlying pupal cuticle. All subsequent development was timed from this point. Table I records the successive characters that appeared during normal development at 25° C. Nearly all these characters are visible in the intact animal when examined under the dissecting microscope as described above.

TABLE II

Critical doses causing normal or abnormal development of cynthia pupae*

Materials (1)	Dose (μg.) provoking:			
	(2) Early development only	(3) Normal development	(4) Abnormal development	(5) Pupal characters
Cyasterone	0.1	0.2–0.3	0.6	2.5–5
Ponasterone B	1	2.5	5	5–10
Ponasterone A	1	2.5	5–10	10
Ponasterone C	2.5	5	10	>10
α-Ecdysone	2.5	5	40	>80
β-Ecdysone	5	10	20	>80
Inokosterone	5	10	20	>20

* Each material was injected in 50 μl. of 10% isopropanol.

RESULTS

1. *Activities of synthetic α-ecdysone and six phytoecdysones*

Each dose of each material was assayed on ten to fifteen normal pupae and five to ten brainless pupae. The developmental responses of the two types of pupae were indistinguishable; therefore the two sets of data have been combined in the presentation of the results in Table II.

In column 1 of this table the seven materials are listed in descending order of endocrine activity. Column 2 records the doses which ordinarily provoked the retraction of the wing epidermis but no further development beyond the first day. Column 3 identifies the doses which usually caused the formation of normal moths. Column 4 records the minimal doses that usually provoked the formation of abnormal moths. Column 5 records the minimal doses that caused certain of the abnormal moths to retain one or more patches of pupal cuticle.

Clearly, in terms of all these criteria, cyasterone is in a class by itself, its activity being ten times that of ponasterone A or B and twenty times that of α-ecdysone.

2. *Variations in the developmental response*

As mentioned above, all seven materials were able to provoke a wide range of developmental responses as dictated by the dose, the specific activity of the injected material, and variations in the intrinsic sensitivities encountered among otherwise homogeneous groups of test pupae. As indicated in column 3 of Table II, critically low doses of each material were able to cause normal development which obeyed the time-table described in Table I and diagrammed as the top line of Figure 5. Half this dose ordinarily caused development to begin and then stall within one day. And for all materials except α-ecdysone, abnormal development was usually provoked by a doubling of the critical dose.

Unlike the other six materials, α-ecdysone caused abnormal development only when administered in extremely high doses (40–80 μg.). Even then the abnormalities were less than those produced by far lower doses of the other materials.

3. *Assays on isolated pupal abdomens*

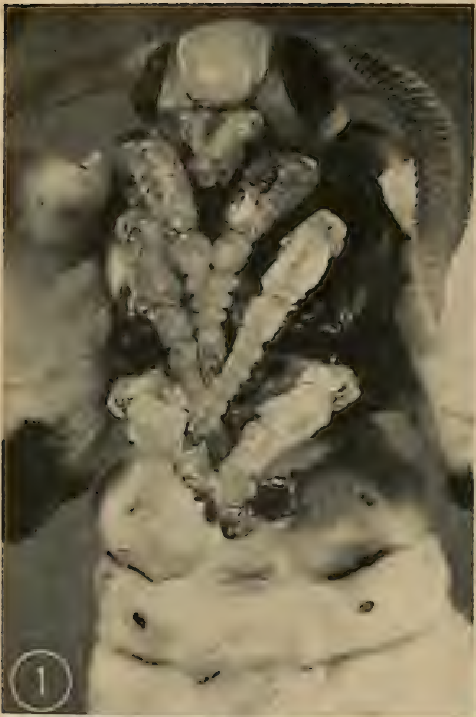
The ecdysone activity of all seven materials was confirmed by injecting 20 μg. into each of a total of 24 isolated pupal abdomens. Three of the six abdomens that received α-ecdysone underwent full and complete adult development; the other three showed no detectable response. Of the three abdomens injected with inokosterone, one failed to respond and two underwent only early development. The other five materials caused all 15 preparations to form abnormal adult abdomens.

4. *Abnormal development*

When administered at or above the dose levels recorded in column 4 of Table II, all seven materials usually caused faulty development. Abnormalities ranged from barely detectable defects in wing pigmentation to the production of the extremely aberrant creatures illustrated in Figures 1–4.

Abnormal development was routinely associated with remarkable acceleration of developmental events during the first few days after the injection. This fact is illustrated in Figure 5 where the upper horizontal line records the sequence of events when normal development was triggered by the injection of a low dose of cyasterone (0.2 μg.). The lower horizontal line illustrates the timing of these same events when extremely abnormal development was provoked by excessive cyasterone (10 μg.).

We first observe that the "latent period" between the injection and the initiation of wing retraction was reduced from 4 to 2 days by the excessive hormone. Extreme derangement in development was then signalled by an excessive retraction of the epidermis from the pupal cuticle. Thus, by the end of the first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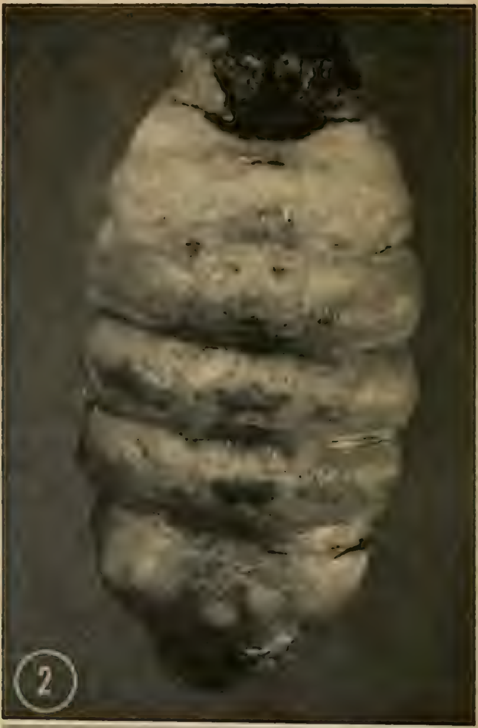
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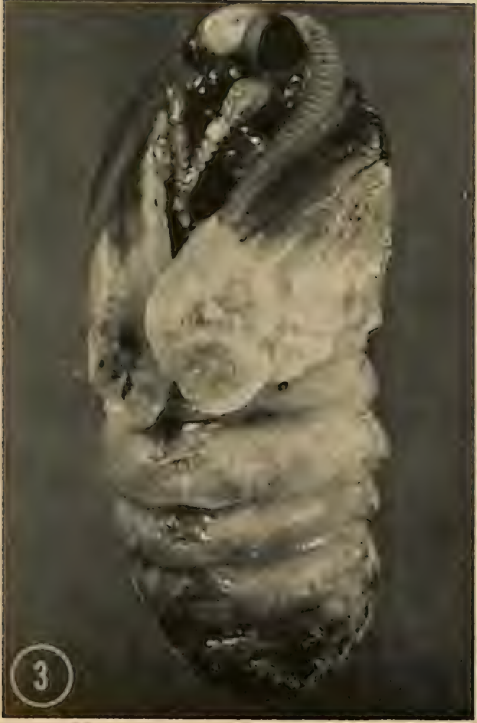
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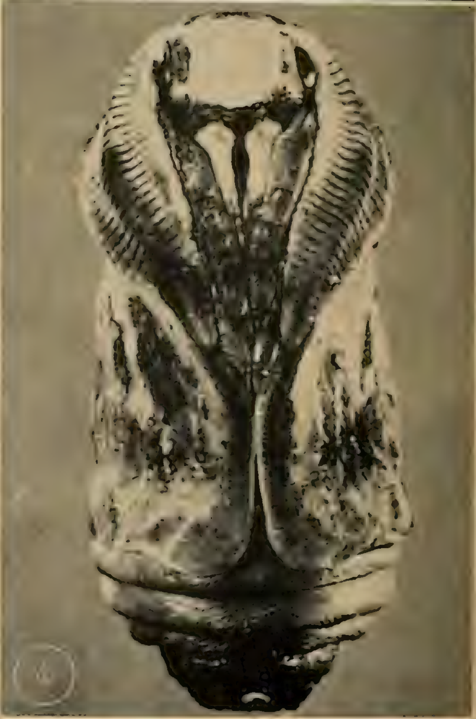
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day, the epithelia of the forewings had retracted to the base of the pupal wing sheaths to form compacted sheets containing convoluted tracheae. The next indication of abnormal development was the precocious formation of ommatidia and the deposition of cuticle and pink eye pigment. These events, which normally occur on the 7th and 8th day, were accelerated to as early as the 3rd day and, in fact, took place before the epithelia of the eyes had expanded to normal size. The more precocious the deposition of cuticle and eye pigment, the more abnormal were the moths that ultimately formed.

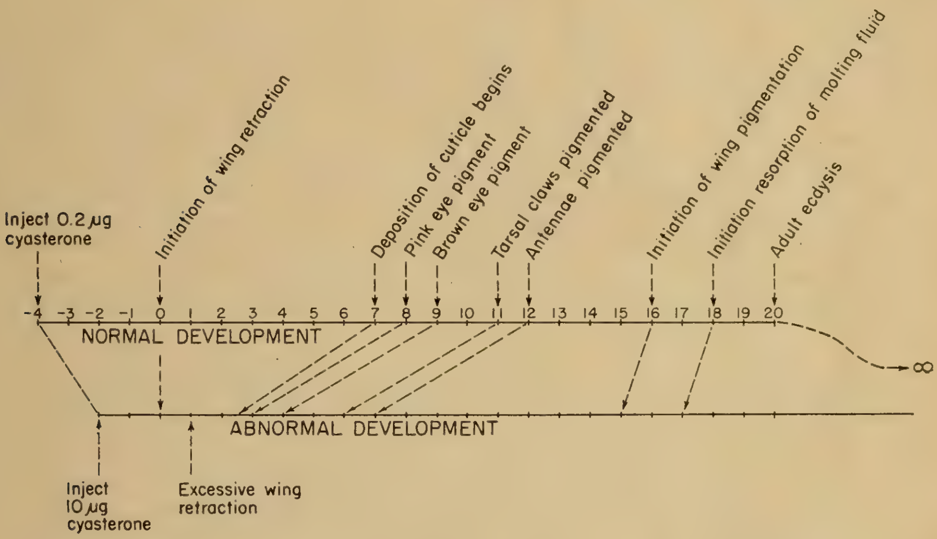
Inject 0.2 μg cyasterone
Initiation of wing retraction
Deposition of cuticle begins
Pink eye pigment
Brown eye pigment
Tarsal claws pigmented
Antennae pigmented
Initiation of wing pigmentation
Initiation resorption of molting fluid
Adult ecdysis
-4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
NORMAL DEVELOPMENT
∞
ABNORMAL DEVELOPMENT
Inject 10 μg cyasterone
Excessive wing retraction

FIGURE 5. The top line records the time-table of successive developmental events at 25° C. when normal development of a cynthia pupa is provoked by the injection of a physiological dose of ecdysone (in this case 0.2 μg. of cyasterone). The lower horizontal line illustrates the timing of these same events when extremely abnormal development is provoked by the injection of an excessive dose of phytoecdysone (in this case 10 μg. of cyasterone).

5. *Telescoping of early development*

As diagrammed in Figure 5, excessive hormone drives the early phase of metamorphosis so fast that, counting from the moment of injection, the sequence of events which normally requires 11 days is compressed into as little as 4½. Then, on about the 3rd day of development, the precocious deposition of cuticle locks all epidermal tissues in whatever stage that they have managed to attain.

FIGURE 1. Ventral view of the anterior end of an abnormal cynthia moth formed by a pupa injected with 10 μg. of ponasterone A. (For detailed description see text.)

FIGURE 2. Ventral view of a naked abdomen formed by a pupal abdomen injected with 20 μg. of β-ecdysone.

FIGURE 3. Ventrolateral view of an abnormal moth formed by a pupa injected with 10 μg. of ponasterone B.

FIGURE 4. Ventral view of a "moth" formed by a pupa injected with 20 μg. of cyasterone; the old pupal cuticle has been removed to reveal the extensive areas where pupal cuticle has reformed.

As illustrated in Figure 5, the normal pace is resumed after about the 3rd day of development and the effects of the excessive hormone appear to end at that time. But the damage has already been done—especially in terms of the precocious deposition of new cuticle.

Normal development routinely culminates in a swift series of events: the breakdown of the pupal endocuticle, the resorption of the molting fluid, and the spontaneous ecdysis of the moth. When development was severely deranged, these events never proceeded to completion and in no case did any grossly abnormal animal escape from the pupal exuviae.

The end-results of the injection of high doses of the more active materials are illustrated in Figures 1–4. The isolated abdomen shown in Figure 2 is typical of the developmental response of pupal abdomens to the injection of 5 μg. of cyasterone or 10 μg. of ponasterone A, B, or C. After removing the old pupal cuticle, one finds a naked adult cuticle such as can be prepared from a normal moth by applying melted wax and then peeling away the scales.

Figures 1 and 3 are typical of what happens to pupae injected with excessive doses of the more active phytoecdysones. The eyes are pigmented and faceted but subnormal in size. The head is naked or covered with scant pubescence. The shafts of the antennae are segmented but show little or no formation of bilateral barbs. The legs are abnormally short due to a foreshortening of the femurs. In extreme cases, all the segments are reduced to a series of naked bead-like objects. In some individuals the wings are completely devoid of scales; others show patches of pigmented scales scattered more or less at random. The thoracic tergum is largely naked but otherwise possesses the smooth, tanned sclerites of the moth and the full formation of the articular sclerites at the base of the wings. Often the patagea are the only pubescent areas. The abdomen is covered by a smooth, semi-transparent cuticle of the adult type which is either naked or covered with scant fuzz. The genitalia are undersized but always show some degree of adult development.

6. *Faulty development of the internal organs*

The Malpighian tubules are greatly distended with uric acid and an abnormal amount of this same material is present in the blood and deposited at random throughout the haemocoele. This condition is probably attributable to the failure in the formation of the rectal sac—the normal depository of excretory material including the voluminous meconium. The fat body is friable and resembles that of a normal moth midway adult development. This same resemblance is seen in the thoracic muscles which are always partially differentiated. The hind-gut is short and straight and therefore quite unlike the convoluted organ of a normal moth. The ovaries show early formation of ovarioles, but few or no yolk-rich eggs. The coiled vasa deferens of normal male moths are represented by a pair of short, straight tubes containing elongate bundles of spermatozoa.

7. *The preservation of pupal characters*

As noted in column 5 of Table II, high doses of the three most active compounds (cyasterone and ponasterones A and B) provoked the formation of abnormal moths which sometimes retained one or more patches of rugose cuticle of the pupal type. This phenomenon was clearly correlated with an extreme acceleration of the first few days of development, as documented by the deposition of eye pigment as early as the 60th hour after the initiation of wing retraction. The occurrence and localization of the rugose areas were capricious. Favorite sites included the thoracic tergum, the antennae, the base and anterior margins of the forewings, and the dorsal and ventral midlines of one or more abdominal segments.

The most extreme instance of the preservation of pupal cuticle is illustrated in Figure 4—a unique individual derived from a pupa injected with an enormous dose of cyasterone (20 μg.). Tanned or untanned pupal cuticle has been retained over extensive areas of head, thorax, and abdomen, thereby mimicking the developmental response one would anticipate, not from ecdysone, but from juvenile hormone.

8. *Injections after the initiation of adult development*

Two hundred chilled cynthia pupae were removed from cocoons and placed at 25° C. After the first ten initiated adult development, the rest were examined at least once a day to detect the initiation of adult development, as signalled by the first signs of the detachment and retraction of the epidermis from the pupal cuticle. Individuals were injected with 10 μg. of cyasterone at 8, 16, 24, 48, and 72 hours after the initiation of wing retraction. In parallel experiments, the individuals were injected with 100 μg. of a preparation of synthetic juvenile hormone (Law *et al.,* 1966) dissolved in 10 μl. of an active extract of Cecropia juvenile hormone (Williams and Law, 1965).

When the cyasterone injection was delayed until the 72nd hour, all individuals emerged as normal adult moths. Individuals injected at the 48th hour also formed normal moths, except for minor defects in the scale density. When the injection was advanced to the 24th hour, additional abnormalities were evident. For example, the antennae showed the formation of primary barbs with subdivision into secondary barbs only at their tips. Moreover, the wings, as well as the meso- and metathoracic tergites retained large scale-free areas. Additional abnormalities were provoked when the injection was advanced to the 8th or 16th hour. The antennae now showed the formation of undivided primary barbs. The wings were scale-free except along their margins and the thoracic tergum was also naked except for the pubescent patagea. Meanwhile, the abdominal segments showed about one-third the normal density of scales and hairs.

Despite these conspicuous abnormalities, all individuals injected after the initiation of wing retraction formed normal legs, compound eyes, and genitalia; moreover, in these individuals there was never any trace of the re-formation of pupal cuticle. The experiment as a whole demonstrates that excessive ecdysone interferes with events beginning at the moment of injection and terminating during the first 60 hours after the initiation of wing retraction.

In the parallel experiments utilizing juvenile hormone, the impressive difference was the formation, not of naked adult cuticle, but of heavily tanned and rugose pupal cuticle over extensive areas of head, thorax, and abdomen. When the injection was made before or immediately after the initiation of wing retraction, the typical result was the formation of a second pupal cuticle over the entire insect (except for the wings which always remained soft, friable, and essentially devoid of scales). When the injection was postponed for eight or more hours, more and more regions underwent normal adult development. It is not without interest that the full and complete loss of sensitivity to juvenile hormone occurred at substantially the same time as the loss of sensitivity to high doses of ecdysone—namely, on or about the 60th hour after the initiation of wing retraction.

Discussion

1. *Relative activities of ecdysones*

In Table III the results of the present study have been compared with previously reported assays of the same materials on isolated larval abdomens of the fleshfly, *Sarcophaga peregrina* (Ohtaki *et al.*, 1967). When the critical doses are computed per gram live weight, then, as shown in the right-hand column of Table III, the fly assay is found to be 3 to 7 times as sensitive as the cynthia assay for all materials except cyasterone and β-ecdysone. In the fly assay, cyasterone ranked second among the seven ecdysones; in the cynthia assay, it ranked first and, in fact, was an order of magnitude more active than the next most active material. β-Ecdysone ranked third in the fly assay and sixth in the cynthia assay.

Table III

Absolute and relative activities of seven ecdysones in two assays

Substance	Critical dose (μg./g. live wt.)		Cynthia/Sarcophaga
	Cynthia assay*	Sarcophaga assay**	
Cyasterone	0.13	0.29	0.4
Ponasterone B	1.3	0.41	3
Ponasterone A	1.3	0.20	7
Ponasterone C	2.5	0.45	6
α-Ecdysone	2.5	0.63	4
β-Ecdysone	5.0	0.32	16
Inokosterone	5.0	1.15	4

* Critical doses causing 50% of pupae to form normal moths.
** Critical doses causing puparium formation of 50% of larval abdomens.

It will be recalled that far greater differences have been encountered between the Lepidoptera and the muscoid Diptera in their sensitivities to juvenile hormone analogues. Indeed, it would appear that only one of the scores of presently-known materials with juvenile hormone activity for Lepidoptera has any effects on the higher Diptera (K. Sláma, personal communication). Therefore, the surprising fact is the substantial endocrine activity documented for all seven ecdysones in the two kinds of assays.

2. *Development as a function of dose*

We have noted that all the ecdysones provoked normal development when administered at certain critically low doses, and at higher doses caused abnormalities ranging from slight to extreme. These findings are therefore in general accord with those reported by Kobayashi *et al.,* (1967a, 1967b). The Japanese investigators have interpreted the faulty development as the formation of "second pupae (pupal-adult mixtures)" and have likened the abnormalities to those caused by juvenile hormone. In the present investigation, excessive ecdysone caused the production, not of second pupae, but of moths that showed faulty differentiation. Pupal characters were preserved in only an occasional individual and were nearly always limited to one or more localized patches of tanned or untanned pupal cuticle. With rare exception, the developmental aberrations were easily distinguished from those provoked by juvenile hormone (Williams, 1961).

As documented in Figure 5, the effects of excessive hormone begin immediately after the injection and continue until approximately the 60th hour. Derangement in the magnitude of developmental events is documented by the excessive relation of the epidermis. Derangement in the timing of developmental events is exemplified by the precocious formation of new cuticle as early as the 60th hour.

3. *Precocious cuticle formation*

As Wigglesworth (1964) has emphasized, the secretion of cuticle is of special interest because cell divisions and differentiative events, including the extrusion of scales and setae, are possible during only a certain critical phase of the molt beginning with the detachment of the epidermis from the old cuticle and terminating with the initiation of secretion of the new cuticle. Normal development requires that the deposition of cuticle be delayed until all these events have been brought to completion. Manifestly, this need is not met when excessive ecdysone accelerates the deposition of cuticle from the normal 170th hour to as early as the 60th hour. For these several reasons the precocious secretion of new cuticle can account for virtually all the developmental effects observed in integumentary tissues: the scale-free condition of the adult cuticle, the failure in the subdivision of the antennae into barbs, and the subnormal size of the genitalia and compound eyes.

Though this simple explanation suffices for integumentary tissues and organs, it is worth recalling that excessive ecdysone also caused abnormal development of the fat body, the gonads, the midgut, and most other internal organs. Therefore, the precocious secretion of cuticle is the vehicle rather than the cause of aberrant development: cells do not need a cuticle in order to have their metamorphosis upset by excessive ecdysone.

4. *Ecdysone versus juvenile hormone*

The typical effect of juvenile hormone is to cause a pupa to form a second pupa, presumably by opposing the derepression of the genetic information for forming a moth. In effect, the adult "gene-set" does not get turned on and the pupal gene-set does not get turned off.

By contrast, the typical effect of excessive ecdysone is to cause a pupa to form a faulty adult; this we attribute to a telescoping of 11 days of development into as few as 4½. The formation of a naked but otherwise normal adult cuticle gives assurance that the adult gene-set has been turned on and the pupal gene-set has been turned off. Therefore, except in those occasional individuals that re-formed one or more patches of pupal cuticle, excessive ecdysone appears to interfere with the cytological expression and implementation of the genetic instructions for forming a normal moth.

5. *Hypo- and hyperhormonism in insects*

The immediate cause of larval or pupal diapause is the shut-down of ecdysone secretion by the prothoracic glands. In this sense the developmental consequences of hypoecdysonism have for millions of years been put to good use by insects. By contrast, as judged from our experience with the cynthia silkworm, hyperecdysonism—for example the secretion of too much β-ecdysone—would be lethal. Therefore, it is not surprising to find the prothoracic glands non-autonomous and tightly coupled to neuroendocrine controls.

SUMMARY

1. Six ecdysones of plant origin are compared with synthetic α-ecdysone in terms of their ability to provoke normal or abnormal development of diapausing pupae and isolated pupal abdomens of the cynthia silkworm (*Samia cynthia*).
2. The activity of the seven materials ranks in the following order: cyasterone > ponasterone B > ponasterone A > ponasterone C > α-ecdysone > β-ecdysone > inokosterone.
3. All seven materials provoked normal adult development when injected in critical doses ranging from 0.2 μg. (cyasterone) to 10 μg. (inokosterone).
4. All materials except α-ecdysone caused extremely abnormal development when excessive doses were administered to pupae or isolated pupal abdomens. The typical result was the formation of naked or nearly naked non-viable moths which failed to complete adult development.
5. Abnormal development is correlated with a telescoping of the early events in adult differentiation, followed by the precocious deposition of new cuticle.
6. Sensitivity to excessive ecdysone or to juvenile hormone disappears simultaneously at about the 60th hour after the initiation of development.
7. The evidence suggests that excessive ecdysone interferes with the cytological expression and implementation of genetic instructions for forming a normal moth. This mode of action is compared to that of juvenile hormone.

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THE EFFECTS OF SELECTED CHEMICAL AGENTS ON FURROW INDUCTION IN THE EGGS OF ARBACIA PUNCTULATA[1]

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Although furrowing, in sea urchin eggs, does not normally occur until mitotic events have been completed, Zimmerman and Marsland (1956) were able to induce premature furrow formation, in *Arbacia* eggs, prior to mitosis by pressure-centrifugation treatment. These investigators found that furrow induction was associated with both a nuclear factor, released upon nuclear rupture, and a cytoplasmic factor associated with metachromatic β granule material which is released and disintegrates upon breakage of cytoplasmic vacuoles (Marsland *et al.*, 1960; Zimmerman and Marsland, 1960). Normal cleavage activity is known to occur typically after dissolution of the nuclear membrane and has also been linked to metachromatic granule material by various workers (Dalcq *et al.*, 1956; Rebhun, 1958; Mulnard *et al.*, 1959; Kojima, 1959). While the exact mechanism of cleavage remains uncertain, it appears evident that the structural state of the cortical cytoplasm is an important factor in cytokinesis (Marsland, 1956; Zimmerman *et al.*, 1957). Whether or not cytological and biochemical events associated with normal division are similarly involved in experimental furrow induction following pressure-centrifugation is uncertain. Nevertheless, the induced furrowing phenomena may represent a useful tool for the further study of cleavage. The present experiments were designed to establish whether experimental furrow induction could be modified by specific inhibitors which are known to block or retard cytokinesis.

MATERIALS AND METHODS

Living material. *Arbacia punctulata* was obtained from Beaufort, North Carolina, Panama City, Florida, and from the Marine Biological Laboratory, Woods Hole, Mass. The animals from Florida and North Carolina were maintained in artificial sea water (Instant Ocean, Aquarium Systems, Inc., Wickliffe, Ohio). The sea urchins from Woods Hole, Mass., were kept in natural sea water. Eggs were caused to shed by electrical stimulation (Harvey, 1952) and washed three times with filtered sea water prior to insemination. The sperm were obtained by injection of 0.5 *M* KCl. Only those batches of eggs which showed greater than 98% fertilization membranes were used in these studies.

Furrow induction. *Arbacia* eggs were immersed in a variety of chemical agents, at various times, before and after insemination. Some of the experimental eggs

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were exposed to the furrow induction method which consisted of subjecting fertilized eggs of *Arbacia* to a pressure of 12,000 lb./in.^{2} at a centrifugal force of 33,000 *g* for a 5-minute duration (30–35 minutes following insemination). Other experimental eggs were simultaneously subjected to the same centrifugal force at atmospheric pressure. The frequency, stability and position of the induced furrows, in the experimental eggs, were compared with those observed in control eggs which were subjected to the same pressure-centrifugation treatment in the absence of drugs. The pressure centrifuge equipment and method for furrow induction are described more fully in a paper by Zimmerman and Marsland (1960). All experiments were carried out at 20° C.

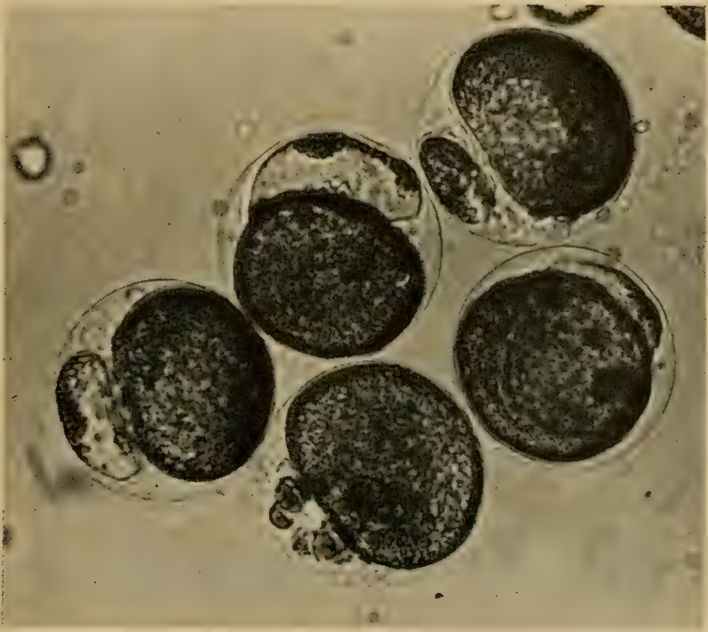

FIGURE 1. Induced furrowing was initiated 30 minutes after insemination (at 20° C.), a time which corresponds to streak stage. The inducing treatment consisted of 5 minutes of pressure-centrifugation (12,000 lb./in.^{2} at 33,000 *g*). Within 5 minutes following treatment approximately 95% of the cells exhibited the furrowing reaction.

Chemicals. Puromycin dihydrochloride and DL-p-fluorophenylalanine monohydrate (p-FPhe) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Dactinomycin (Actinomycin D) was graciously supplied by Merck, Sharp and Dohme. Colcemid (deacetyl-N-methylcolchicine) was graciously supplied by Ciba Company Ltd., Dorval, Quebec. Deuterium oxide (D_2O) with a specific purity of 99.86 *M* per cent was obtained from Bio-Rad Laboratories, Richmond, California. The β mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, New York. Cornin was prepared from rabbit skeletal muscle according to the method of Nisida and Murakami, (1965a). The 2,4 dinitrophenol and the 4,6 dinitro-o-cresol were obtained from Fisher Scientific Company.

Feulgen staining. The eggs were fixed in acetic acid-alcohol fixative (1:3) for 24 hours. The eggs were washed three times with distilled water, then placed on subbed slides and air-dried. The cells were hydrolyzed in 1 N HCl at 60° C. for 10 minutes. The staining period in Schiff's reagent was 20 minutes. Three washings, 10 minutes each, in SO_2 water followed by 3 washings in distilled water removed excess stains. In one experimental series, the eggs were counterstained with 1% light green alcoholic solution prior to dehydration. The eggs were rapidly dehydrated in 80, 95 and 100 per cent alcohol, cleared in xylol, and mounted in Permount.

RESULTS

As was previously established (Marsland *et al.*, 1960; Zimmerman and Marsland, 1960) cleavage furrows can be observed in fertilized eggs of *Arbacia* within a few minutes after the release of pressure, following pressure-centrifugation treatment. The induced furrows are always at right angles to the centripetal axis at the equator or in varying positions from the equator approaching the centripetal pole. Only those eggs that showed a furrow induction frequency of 50% or greater, in which the furrows were maintained for at least 10 minutes after the release of pressure, were used in the experiments (Fig. 1).

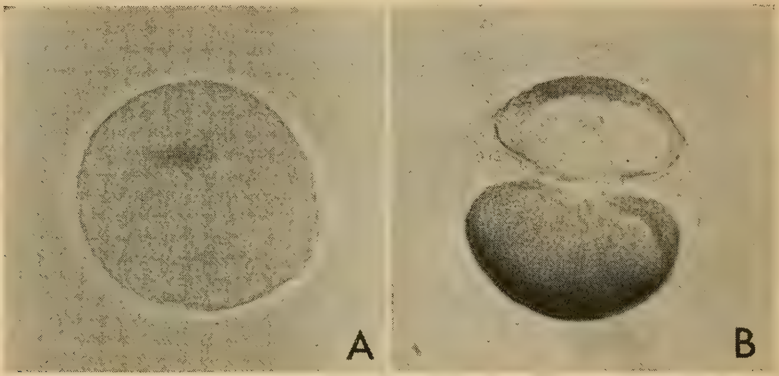

FIGURE 2. (A) Puromycin-treated *Arbacia* egg stained with Schiff's reagent. Cells were incubated in $5 \times 10^{-4} M$ puromycin, 15 minutes before insemination and fixed 2½ hours after insemination. The Feulgen-positive material is accumulated over the nuclear material. (B) Furrow induction in puromycin-treated *Arbacia* egg. The cells were exposed to $10^{-4} M$ puromycin, 15 minutes after insemination and pressure-centrifugation treatment was initiated 30 minutes after insemination.

In an attempt to elucidate the biochemical activities involved in furrow formation, the cells were treated with a variety of metabolic inhibitors prior to the furrow-inducing pressure-centrifugation treatment. In all cases, a division-delaying or blocking concentration of the drugs was used. Observations were made to determine whether these inhibitors would prevent furrow induction and if not whether other parameters such as the frequency, position and stability of the induced furrows were affected.

Metabolic inhibitors of protein and RNA synthesis. Puromycin, a protein inhibitor affecting the synthesis of polypeptide chains, blocks first division in sea urchin eggs in concentrations of 10^{-4} M or greater, at the streak stage (Fig. 2a), if treatment is initiated prior to fertilization, (Hultin, 1961; Rustad and Burchill, 1966; Wilt *et al.*, 1967). When *Arbacia* eggs were immersed in 10^{-4} M puromycin, 15–30 minutes before and 15 minutes after insemination and subjected to pressure-centrifugation, furrow induction was observed in most cases (Fig. 2b). There was no apparent difference between the induced furrow phenomena of cells treated with puromycin before or after insemination. The frequency of furrow induction, in these experimental cells, was sometimes lower than that found in the untreated control cells. However, the position and stability of the induced furrows of puromycin-treated cells and untreated control cells were comparable.

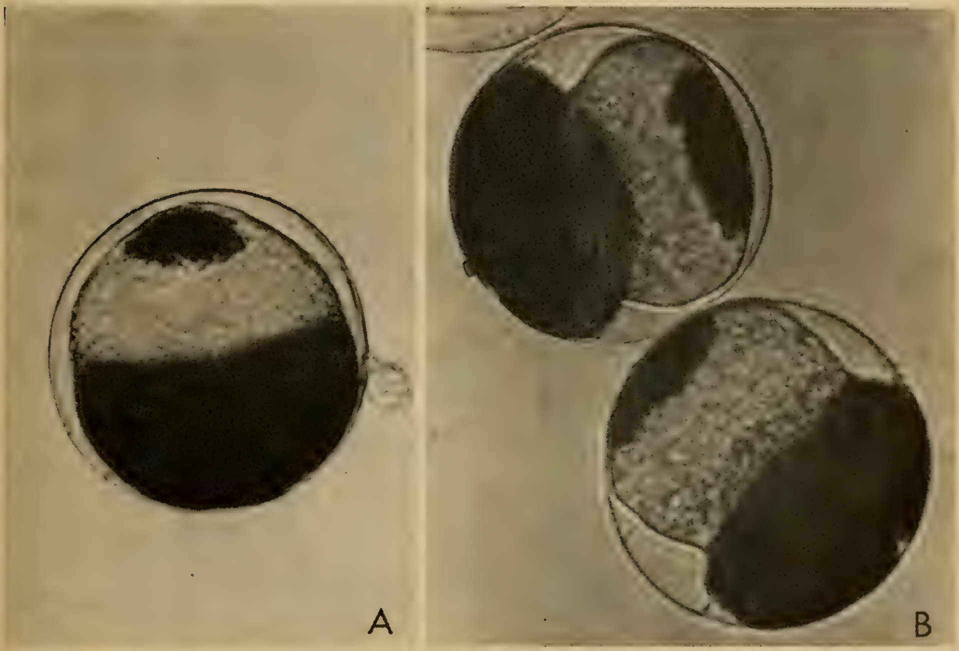

FIGURE 3. *Arbacia* eggs treated with 3.3×10^{-3} M p-fluorophenylalanine 15 minutes after insemination. The eggs were centrifuged 35 minutes after insemination at 33,000 *g* for 5 minutes. (A) Control egg centrifuged at atmospheric pressure. The nucleus is visible in the hyaline zone. The echinochrome pigment is heavily packed in the centrifugal end of the cell. (B) Experimental eggs centrifuged simultaneously but at a pressure of 12,000 lb./in.^{2} The nuclear membrane has been broken and the nucleus is not visible. These cells exhibit furrow induction.

Another protein inhibitor, DL-p-fluorophenylalanine (p-FPhe), has been shown to delay or inhibit cell division in *Tetrahymena*, presumably through the formation of false proteins which do not fit readily into cellular structures (Zeuthen, 1964; Rasmussen and Zeuthen, 1962, 1966). In the present experiments, *Arbacia* eggs were exposed to 3.3×10^{-3} M and 6.6×10^{-3} M p-FPhe, 15 minutes after insemination. In the 3.3×10^{-3} M p-FPhe-treated cells, first division was delayed for

10–15 minutes and some divisions were multipolar. At this concentration, furrow induction was similar to that of the untreated control cells (Fig. 3).

Arbacia immersed in 6.6×10^{-3} *M* p-FPhe, 15 minutes after fertilization, suffered division delays of 30–60 minutes with many multipolar divisions occurring. When these cells were pressure-centrifuged, furrow induction was still evident, albeit considerably reduced in frequency in most cases. However, there was no apparent difference between the untreated control cells and the p-FPhe-treated cells with regard to the position or stability of the induced furrows.

Actinomycin D, an agent known to inhibit synthesis of messenger RNA, does not block early cell divisions or protein synthesis in the sea urchin egg (Gross and Cousineau, 1964). When eggs were exposed to 50 μg./ml. of actinomycin D, 15 minutes before insemination, or 15 minutes after insemination and pressure-centrifuged 30 minutes after insemination, there was no apparent effect on the frequency, stability or the position of induced furrows as compared to those of untreated control cells.

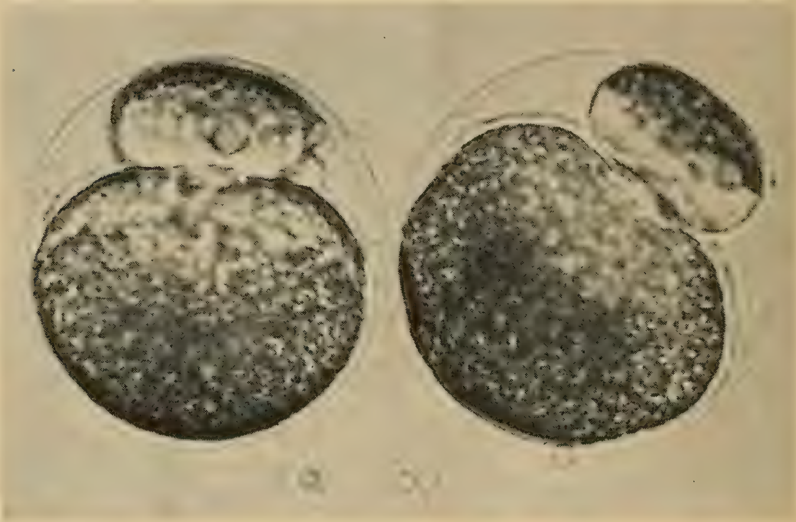

FIGURE 4. Induced furrowing in *Arbacia* eggs treated with 90% D_2O, 5 minutes after insemination. Furrow-inducing pressure-centrifugation treatment was initiated 35 minutes after insemination. This concentration of D_2O blocks pronuclear fusion and subsequent cytokinesis, but did not prevent furrow induction.

Mitotic inhibitors. Heavy water (70%) can block division in sea urchin eggs at various stages of mitosis (Gross and Spindel, 1960; Marsland and Zimmerman, 1963). However, Marsland and Zimmerman (1963) found that 90% D_2O did not block cytokinesis when cells were treated immediately preceding furrowing. In addition, they observed premature furrowing in the D_2O-treated eggs, although not as frequently as in the controls.

In the present experiments, eggs were placed into 70% and 90% heavy water 15 and 20 minutes after insemination and pressure-centrifuged 30–35 minutes after insemination. The results are in agreement with Marsland and Zimmerman

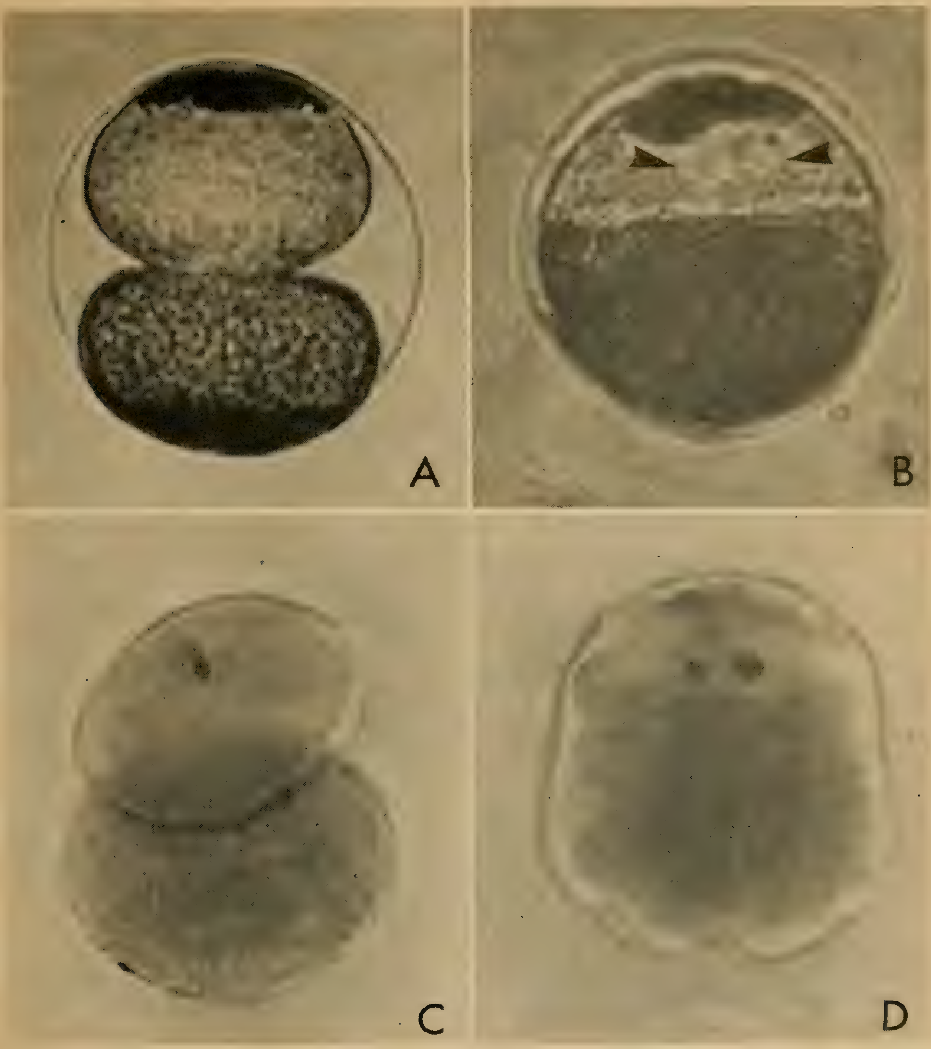

FIGURE 5. *Arbacia* eggs treated with colcemid ($2.7 \times 10^{-4} M$) 5 minutes after insemination, before pronuclear fusion occurred. The cells were centrifuged at 35 minutes after insemination at a force of 33,000 *g* for 5 minutes. (A) and (C) show furrow induction following centrifugation at a pressure of 12,000 lb./in.^{2} The cell in (C) has been Feulgen-stained; the Feulgen-positive material is visible in the lighter blastomere. In (B) and (D), the cells were centrifuged simultaneously but at atmospheric pressure. The arrows in (B) point to the non-fused pronuclei, a consequence of colcemid treatment. The cell in (D) has been Feulgen-stained and the two pronuclei are seen as Feulgen-positive material.

(1963) in that the D_2O-treated eggs exhibited premature furrowing but the frequency of furrow induction, in these eggs, was usually lower than that found in the untreated controls. There was no obvious difference between the experimental and the control eggs as to the stability and position of the induced furrows.

In other experiments, eggs were put into 70% and 90% D_2O, five minutes after insemination. Feulgen-stained samples fixed 45 minutes after insemination indicated that the pronuclei did not fuse. When these eggs were pressure-centrifuged 35 minutes after insemination a stable furrow induction was observed but the percentage of cells which induced furrows was lower than that found in the untreated controls (Fig. 4).

Colcemid, a derivative of colchicine, can inhibit first division in sea urchin eggs by blocking pronuclear fusion (Zimmerman and Zimmerman, 1967) as well as by disorganizing the mitotic apparatus (Sauaia and Mazia, 1961). Cells were exposed to blocking concentrations of colcemid (2.7×10^{-4} *M*) five minutes after insemination in order to block pronuclear fusion. When these cells were pressure-centrifuged, 35 minutes after insemination, the induced furrows were similar to those found in untreated cells (Fig. 5A). The centrifuged but non-pressurized cells treated with colcemid displayed two pronuclei in the hyaline zone (Fig. 5B). Feulgen-stained samples of such eggs revealed the male and female pronuclei at varying distances from one another (Fig. 5C, D). The furrow induction of cells treated with colcemid (2.7×10^{-4} *M*) 15 minutes after insemination (following pronuclear fusion) was also similar to that of the untreated control cells.

Thiol agent. Experiments employing mercaptoethanol, a substance having a readily available source of thiol groups, have demonstrated that gelated structures within sea urchin eggs, such as the mitotic apparatus (Mazia and Zimmerman, 1958) and the cortical plasmagel (Zimmerman, 1964), are markedly disorganized by the addition of mercaptoethanol. When eggs were placed into 0.09 *M* mercaptoethanol 15–20 minutes after insemination and subjected to pressure-centrifugation the frequency of induced furrows was comparable with or greater than that of the untreated control cells. However, many of the induced furrows tended to abort within 3–5 minutes. The position of the induced furrows appeared to be most sensitive to mercaptoethanol since, following treatment, the majority of furrows would occur at the equatorial region of the cell regardless of the position of the furrows of untreated control cells. When the concentration was lowered to 0.075 *M* (also a blocking concentration) the frequency and stability of the induced furrows were comparable with those of untreated control cells, except for the tendency of the induced furrow position to shift to the equatorial region of the cell.

Inhibition of oxidative phosphorylation. Cornin, a polypeptide, has been shown to be a potent inhibitor of oxidative phosphorylation in sea urchin eggs and mammalian cells (Nisida and Murakami, 1965b). When eggs were treated with 10^{-6} g/ml. cornin, 10 minutes before insemination, 87% of the cells divided after one hour. When these cells were subjected to pressure-centrifugation 35 minutes after insemination, the induced furrows were similar to those found in untreated cells except that the frequency of furrow induction was slightly lower. When the concentration of cornin was increased 10-fold to 10^{-5} g./ml. only 21% of the cells were in the 2–4-cell stage after two hours and all the cells were broken after pressure-centrifugation. It was also found that many of the cornin-treated centrifuged cells became abnormally swollen.

The effects of other oxidative phosphorylation inhibitors, 2,4-dinitrophenol (DNP) and 4,6-dinitro-o-cresol (DNC), on experimental furrow induction were also observed. *Arbacia* eggs treated five minutes after insemination with 10^{-5} *M* DNP or 10^{-5} *M* DNC showed 6% division and 20% division, respectively, in the 2–4-cell stage, after 20 hours. Control cells were in the free-swimming blastula stage at this time. When similarly treated cells were pressure-centrifuged 35 minutes after insemination there was a slight reduction in the frequency of induced furrows.

Discussion

In general the results indicate that experimental furrow induction following pressure-centrifugation is not blocked by chemical agents which inhibit mitosis and cytokinesis. Moreover, the fusion of the pronuclei does not appear to be a necessary prerequisite for the furrow induction phenomena.

The experiments with inhibitors of RNA and protein synthesis suggest that the production of new messenger RNA and the synthesis of new protein are not essential for the furrow induction phenomena. The results with actinomycin D are not surprising in view of the studies of Gross and Cousineau (1964) who demonstrated that synthesis of new RNA does not occur until some 4–6 hours after insemination, in sea urchin eggs. The puromycin effects on furrow induction give some indication that protein synthesis may exert some influence on the ability of cells to form experimental furrows since, following this treatment, the number of cells which formed furrows was reduced. Recently, Wilt *et al.* (1967) suggested that protein synthesis of "catalytic components" may be important in the functioning of cell division.

DL-p-fluorophenylalanine, a competitive inhibitor of phenylalanine, caused division delays and multipolar furrow formation at the two concentrations employed. However, the high concentration (6.6×10^{-3} *M*), alone, modified the experimental furrow induction by reducing the frequency of furrow formation. Zeuthen and co-workers (Zeuthen, 1964; Rasmussen and Zeuthen, 1962, 1966), studying the effects of p-fluorophenylalanine on *Tetrahymena,* proposed that p-FPhe is incorporated into proteins which are essential for cell division, and the synthesis of such p-FPhe proteins ("false proteins") interferes with the furrowing reaction. If a comparable situation occurs in sea urchin eggs, the production of "false proteins" may interfere with the formation of structural protein involved in the cleavage process. This hypothesis might explain the significant division delay and multipolar furrow formation following p-FPhe treatment without an immediate division block as well as the definite reduction in frequency of experimental furrow induction.

The experiments with mitotic inhibitors (colcemid, mercaptoethanol and heavy water) show different effects on furrow induction with each inhibitor. This may suggest that the observed modifications of furrow induction reflect an interference more with cortical gel structure than with mitotic apparatus structure. Colcemid did not appear to have any effect on experimental furrow induction yet it is known to specifically disorganize the mitotic apparatus in sea urchin eggs (Sauaia and Mazia, 1961). Recently, Taylor and co-workers (Borisy and Taylor, 1967a, 1967b; Shelanski and Taylor, 1967) have suggested that colchicine (the parent compound of colcemid) acts by binding to a protein subunit of microtubules, thus preventing the assembly of subunits into microtubule structure.

Deuteration appears to reduce the capacity of sea urchin eggs to form experimental furrows. Marsland and Zimmerman (1963) suggest that this may be a consequence of nuclei and β granule resistance to centrifugal breakage as well as interference with normal bonding in protein structure.

It is difficult to assess the relationship of the cortical gel structure of the cell to the experimental furrowing reaction. Mercaptoethanol treatment which weakens the cortical plasmagel (Zimmerman, 1964) does not reduce the capacity of the cells to form experimental furrows but results in less stable furrows. The reduced stability of the furrowing reaction in mercaptoethanol-treated cells may be a consequence of interference with the formation of stable disulfide bonds which are essential for maintenance of structural proteins involved in furrow maintenance. It is also interesting to note (although difficult to interpret) that the position of the furrow in these cells was usually at the equator.

It is known that cornin, DNP and DNC act as uncouplers of oxidative phosphorylation and interfere with the orderly formation of energy-rich phosphate compounds (Nisida and Murakami, 1965a; 1965b; Simon, 1953; Zimmerman *et al.*, 1958). These agents block cell division at specific concentrations in sea urchin eggs as shown in the present work and by others (Clowes, 1951; Nisida and Murakami, 1965a, 1965b), and in *Tetrahymena* (Hamburger and Zeuthen, 1957). The present study shows that these inhibitors of oxidative phosphorylation do not exert any significant effect on furrow induction. This would suggest that the formation of experimental furrows is not dependent upon oxidative phosphorylation for its energy source.

SUMMARY

Following high speed pressure-centrifugation treatment, fertilized eggs of *Arbacia punctulata* exhibit a furrowing reaction prior to cytokinesis. Fertilized eggs were incubated in metabolic and mitotic inhibitors and the effects of these agents on frequency, stability and position of the induced furrows were observed. Puromycin and p-fluorophenylalanine (inhibitors of protein synthesis), in concentrations which inhibit cytokinesis in control cells, did not prevent the induced furrowing reaction, although the frequency of furrow induction was reduced. Actinomycin D (an inhibitor of mRNA synthesis) had no effect on furrow induction. Heavy water and colcemid, at concentrations which block pronuclear fusion and cell division, did not block furrow induction. Mercaptoethanol treatment (at concentrations which block cytokinesis) did not reduce the frequency of induced furrows but resulted in less stable furrows and in shifting of the plane of furrow to an equatorial position. Agents that block oxidative phosphorylation (cornin, dinitro-o-cresol, dinitrophenol) did not block the furrowing reaction, although first division was markedly delayed. The results are discussed in terms of the effects of mitotic and cytokinetic inhibitors on experimental furrow induction.

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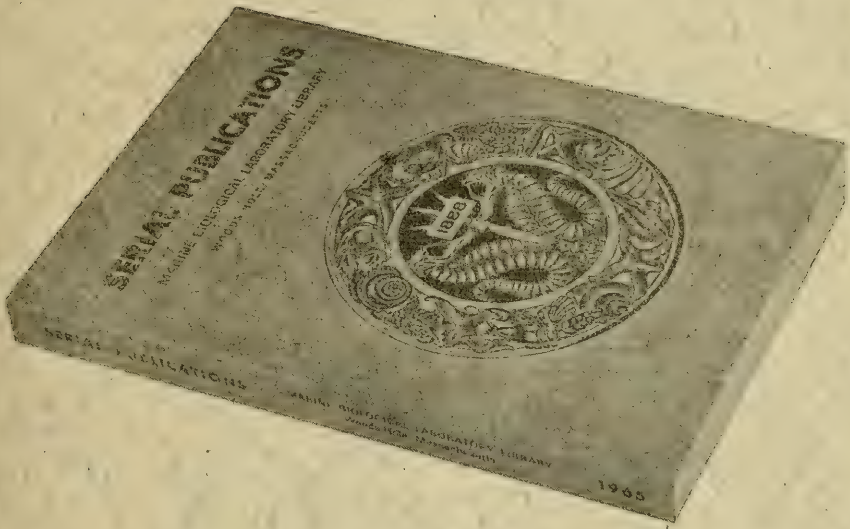
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CONTENTS

Page

BARNWELL, FRANKLIN H.
Comparative aspects of the chromatophoric responses to light and temperature in fiddler crabs of the genus Uca 221

BORN, JOHN W.
Osmoregulatory capacities of two caridean shrimps, Syncaris pacifica (Atyidae) and Palaemon macrodactylus (Palaemonidae) 235

CAMPBELL, RICHARD D., AND FRANCE CAMPBELL
Tubularia regeneration: Radial organization of tentacles, gonophores, and endoderm 245

CLARK, MARY E.
A survey of the effect of osmotic dilution on free amino acids of various polychaetes 252

FARLEY, ROGER D., AND JAMES F. CASE
Perception of external oxygen by the burrowing shrimp, Callianassa californiensis Dana and C. affinis Dana 261

HUMPHRIES, A. A., JR., SALLIE B. FREEMAN AND WILTON M. WORKMAN
Sialic acid in egg capsules and oviducts of the newt, Notophthalmus viridescens 266

JONES, MEREDITH L.
On the morphology, feeding, and behavior of Magelona sp. 272

KONIJN, THEO M.
Chemotaxis in the cellular slime molds. II. The effect of cell density 298

MORSE, M. PATRICIA
Functional morphology of the digestive system of the nudibranch mollusc Acanthodoris pilosa 305

RUGH, ROBERTS, AND CSILLA SOMOGYI
Hematological recovery of mouse following fetal x-radiation 320

SMITH, ROY EDGAR
Experimental evidence for a gonadal-fat body relationship in two teiid lizards (Ameiva festiva, Ameiva quadrilineata) 325

STUNKARD, HORACE W.
The asexual generations, life-cycle, and systematic relations of Microphallus limuli Stunkard, 1951 (Trematoda: Digenea) 332

WILLIAMS, CARROLL M.
Ecdysone and ecdysone-analogues: Their assay and action on diapausing pupae of the cynthia silkworm 344

ZIMMERMAN, SELMA B., TETUHIDE H. MURAKAMI AND ARTHUR M. ZIMMERMAN
The effects of selected chemical agents on furrow induction in the eggs of Arbacia punctulata 356

Volume 134 Number 3

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A COMPARATIVE STUDY OF P^{32} UPTAKE BY WHOLE ANIMALS AND ISOLATED BODY REGIONS OF THE SEA CUCUMBER HOLOTHURIA ATRA

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Many marine organisms have demonstrated the ability to remove phosphorus compounds containing the radionuclide P^{32} from solution. It was first established by Chu (1946) that soluble organic phosphorus compounds in the sea could be utilized directly by algal cultures. Phosphorus requirements of the algae were satisfied when a supplement of glycerophosphate or phytin was added to culture flasks. Rigler (1961) demonstrated an uptake of 2.2×10^{-3} μ-gram PO_4/animal/hour by *Daphnia magna*, a fresh-water microcrustacean. This uptake rate was significantly greater than the uptake rate of sterile animals (pre-incubated in an antibiotic solution). Rigler concluded that epizootic bacteria were largely responsible for the apparent uptake of phosphorus in these experiments, but he is uncertain whether this phosphorus becomes available to *D. magna.*

Further evidence for the fate of phosphorus compounds was provided by studies with both sterile and non-sterile *Lembos intermedius,* a gammaridean amphipod (Johannes, 1964). In contrast with the results reported by Rigler (1961), Johannes reports that sterile individuals of this marine crustacean accumulated P^{32} in a linear fashion. Each gram of amphipod absorbed an amount of P^{32} per hour which was equivalent to the P^{32} contained in 7 ml. of solution. Therefore, it appears that phosphorus compounds are accumulated to some degree in the case of the marine amphipods investigated by Johannes. Stephens (1964) demonstrated a direct relationship between uptake of glycine-C^{14} by *Nereis limnicola* and the percentage of sea water in the ambient medium. The rate of removal of the labeled amino acid was found to decrease significantly with a decrease in salinity.

The uptake of at least amino acids from solution by marine forms seems to be related to the osmotic conditions of the experimental incubating solution. Since the fresh-water nature of *D. magna* may be a factor influencing the very low P^{32} uptake by sterile animals, a repetition of the sterilizing techniques of Rigler (1961) on a marine organism, followed by direct exposure to labeled phosphorus, may

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yield additional information about osmotic effects on observed nuclide uptake. In view of the conflicting reports of Rigler (1961) and Johannes (1964) regarding the acccumulation of labeled phosphorus by crustaceans, as well as the salinity relationship shown by Stephens (1964) to exist in marine polychaetes, it is of interest at present to determine whether epizootic and/or endozoic bacteria do, in fact, contribute to the observed phosphorus uptake by marine organisms.

The grossly observed phenomenon of phosphorus uptake by an organism may be treated as the sum of several simultaneous, interdigitating processes. It is of interest, therefore, to assess the contributions which each of the pathways into and out of the body makes to the total observed uptake. An examination of these pathways yields information on uptake and release kinetics as well as data pertaining to net phosphorus retention.

In order to compare the gross uptake and distribution of phosphorus in an animal exposed to sea water containing the isotope P^{32} with the uptake of phosphorus by isolated portions of an intact animal, a means of restricting and partitioning the animals was devised. There are at least three pathways by which inorganic phosphorus may enter the body of a living specimen of *Holothuria atra*. The mouth, representing the entrance for the alimentary tract, appears to be an adequate pathway for entrance of dissolved materials. The respiratory tree complex, being a center for exchange of gases, is potentially active in the exchange of chemical compounds. The integument represents a third region by which the holothurian could conceivably gain an energy source from dissolved material in the ambient medium. An incubating chamber was devised (Ahearn, unpublished data) that practicably separated an animal into an oral region, an integumental region, and an anal region. With each region sealed off from each adjoining region so that no exchange of materials occurred between them, the dynamics of phosphorus uptake by the different portions of the body of the test animal were examined.

The present study was undertaken in the pursuit of three objectives. (1) To follow the gross uptake and subsequent distribution of P^{32} in intact unrestricted specimens of *H. atra* exposed to the enriched sea-water medium. (2) To evaluate the contribution to the above gross uptake by each of three possible pathways of phosphorus entry—the mouth, the integument, and the respiratory tree complex—through the use of a split tank that effectively partitioned an animal into these three regions, with a minimal effect upon normal physiology. (3) To determine the possible effects of epizootic and/or endozoic bacteria on the observed gross P^{32} uptake by *H. atra*, by incubating the holothurians in an antibiotic solution before their introduction to the P^{32} sea-water medium and subsequently following the phosphorus kinetics.

The author wishes to thank Dr. Sidney J. Townsley, Department of Zoology, University of Hawaii, for suggestions leading to the design and utilization of the split tank and for his consistent patience and encouragement throughout the duration of this study.

MATERIALS AND METHODS

Specimens of *H. atra* used in this study were collected from the Queen Surf area of Oahu, Hawaii. Prior to each experiment animals were held in recirculating sea water for two to seven days for the gut contents to be cleared. The wet weights of animals used in this study ranged from 75 to 300 grams. Phosphorus was supplied to the test animals as carrier-free phosphoric acid (H_3PO_4) added in measured quantities to HA Millipore-filtered sea water (filter size 0.45 μ).

The first series of experiments were designed to demonstrate the gross uptake of P^{32} by unrestricted specimens of *H. atra*. This uptake provided an estimate of gross nuclide removal from the ambient medium by all phosphorus uptake pathways in the animal. A total of 15 animals were incubated for specific times, each in separate containers with the enriched sea-water medium. The disappearance of P^{32} from the medium was followed by removing triplicate 0.5-ml. aliquots from each of the 15 receptacles at selected time intervals during incubation. Three animals were removed from their respective chambers containing the incubation medium after 6, 12, 24, 30, and 48 hours. They were thoroughly rinsed in sea water containing no isotope so that any P^{32} adhering to the tissues might be removed. The animals were divided into two fractions and counted for P^{32} activity. These fractions were the visceral organs (gut, gonads, left and right respiratory trees and rete mirabile) and the integument. The tissues were partially dehydrated (100° C. for 24 hours) and later ashed in a muffle furnace at 600° C. for 24 hours. The ash weight was obtained and aliquots were plated on preweighed aluminum phanchets. Coelomic fluid samples were not withdrawn from specimens for this initial estimate of gross uptake of P^{32}, but were later taken from animals that were exposed to the nuclide in the split tank for a better evaluation of phosphorus distribution throughout these animals. The P^{32} activity of the ash aliquots was determined by using a Nuclear-Chicago Decade Scaler and Geiger-Mueller Detector in combination with an automatic planchet changer. Appropriate corrections were made for background radiation, detector efficiency, and half-life decay. Results were obtained as counts per minute per gram of ash of integument or visceral fraction. The resulting activity was reported as concentration ratios according to the following conventions:

$$\text{C.R.} = \frac{\text{cpm/gm. ash}}{\text{initial cpm/ml. medium}} \quad \text{and} \quad \text{C.R.} = \frac{\text{cpm/gm. ash/hour}}{\text{initial cpm/ml. medium}}.$$

The second concentration ratio is corrected for incubation time.

To assess the contribution to phosphorus uptake made by the three pathways into the holothurian body, a series of experiments were designed utilizing a split tank (Fig. 1). This rectangular 5-liter Plexiglas tank was divided by two baffles into three chambers; each end chamber held 2 liters, and the central chamber held 1 liter. Surgical rubber gloves served as the dividing membranes and held an intact animal so that its oral and anal regions projected into each of the end chambers, while a small area of integument was isolated in the central chamber. A glove was pulled over a thin rectangular plastic frame so that the fingers were directed vertically above the tank and the thumb extended horizontally into the respective end chamber. The ends of the fingers and thumb were removed with a pair of scissors. A small vertical slit was made in the portion of the glove which faced the central chamber so that a specimen of *H. atra* could be pushed through the thumb and into the central chamber. With a glove similarly arranged on the second plastic frame an animal (of an appropriate length) could be slipped from one end chamber, through two gloves and into the second end chamber. In this manner the gloves served as restraining devices, as well as partitions that isolated the mouth, a portion of the central integument, and the anal region of the animal. The gloves were sealed to the baffles by means of silicon gel and rubber tubing. This measure prevented any isotope leakage between chambers during the experimental trials. Before each experiment was initiated, a check was made for leakage between the chambers with an animal in place. Five experiments, each for an incubation period of 6 hours, were performed according to the following schedule: two split-tank animals were exposed to P^{32} in the central chamber and filtered sea water in the oral and anal chambers; two animals were exposed to the isotope in the anal chamber and "cold" sea water in the remaining chambers; and a single animal was exposed to P^{32} in the oral chamber and filtered sea water in the other two chambers.

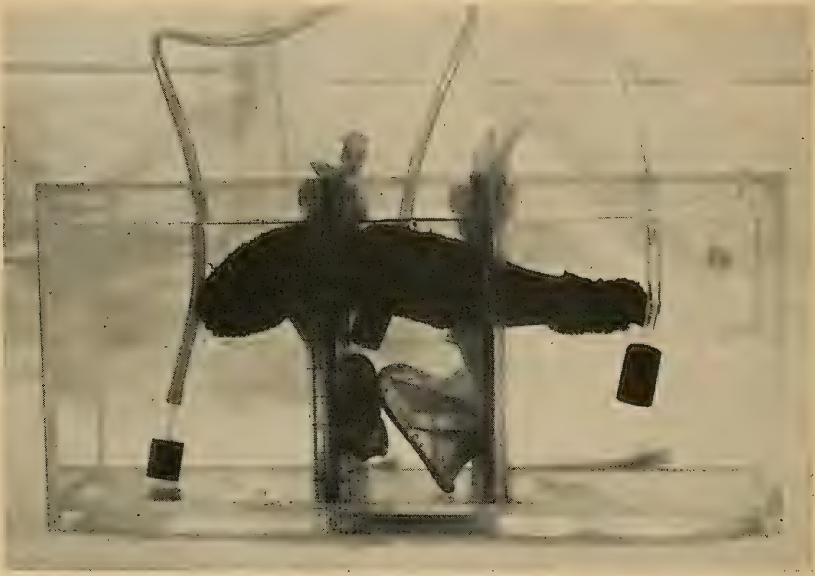

FIGURE 1. Plastic split tank with specimen of *Holothuria atra,* illustrating typical experimental technique of dividing the animal into an oral, integumental, and anal region. Surgical rubber gloves fitted around rectangular plastic frames served as the dividing membranes. The end chambers each held a total volume of 2 liters while the central chamber's capacity was 1 liter. Air was supplied independently to each chamber.

During each experiment triplicate aliquots (0.5-ml.) of the medium from each chamber of the tank were removed hourly. These were dried on planchets for counting. Such samples indicated not only the decrease of radioactivity from the chamber initially containing activity, but also the appearance of activity in each of the other chambers; that is, leakage across the membrane or outflow from the animal. At the end of 6 hours of incubation with the isotope in the respective chamber, the animals were removed from the split tank and washed with filtered sea water to remove adsorbed P^{32}. Immediately after washing, an integumental puncture was performed (standard medical syringe equipped with a stainless steel 18-gauge needle) to remove a sample of the coelomic fluid. Triplicate 0.5-ml. samples of the coelomic fluid were dried under infrared light and assayed for P^{32} in the manner described for tissue fractions. The remainder of the coelomic fluid was withdrawn and the total volume measured. Each organ of the viscera was removed from the split-tank animals and independently assayed for its phosphorus activity. This differs from the previous experiments assessing gross uptake where all the visceral organs were counted for P^{32} activity as one fraction. The phosphorus content of the integument fraction was also determined. Standard dehydrating, ashing and counting procedures were used as previously described. By separating the organs and coelomic fluid in this manner, a percentage of total body activity could be ascribed to each tissue component and some measure of its phosphorus uptake could be obtained.

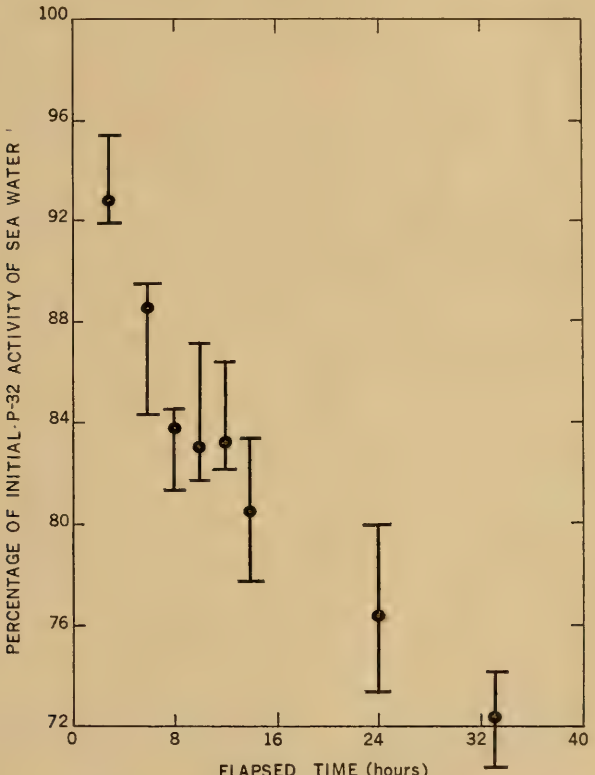

FIGURE 2. Relative decrease in phosphorus activity from the enriched sea-water medium by unrestricted specimens of *H. atra* exposed to P^{32} for selected time intervals. Each point represents the mean value of the activity in the media of three separate animals exposed to the nuclide for the same amount of time. Ranges are indicated by vertical lines.

The final experiment of this study was designed to investigate the possible influence of epizootic and/or endozoic bacteria in the observed uptake of phosphorus by *H. atra*. The general procedure followed for reducing the holothurian bacterial population was adapted from that used by Rigler (1961) to reduce bacterial flora on *Daphnia magna*. Six animals were held in sea water for three days prior to initiation of the experiment so that the gut contents might be eliminated. Ten liters of a pre-incubation medium were prepared by filtering sea water (HA Millipore Filter, 0.45 μ) and adding 100 μ-grams streptomycin and 100 units penicillin per ml. The animals were placed for two 90-minute periods in this antibiotic solution. During the 12-hour interim between treatments, the animals were retained in 10 liters of aerated Millipore-filtered sea water. Following the second pre-incubation, each animal was placed in an individual receptacle containing 3 liters of filtered sea water to which P^{32} bound as phosphoric acid was added. Ani-

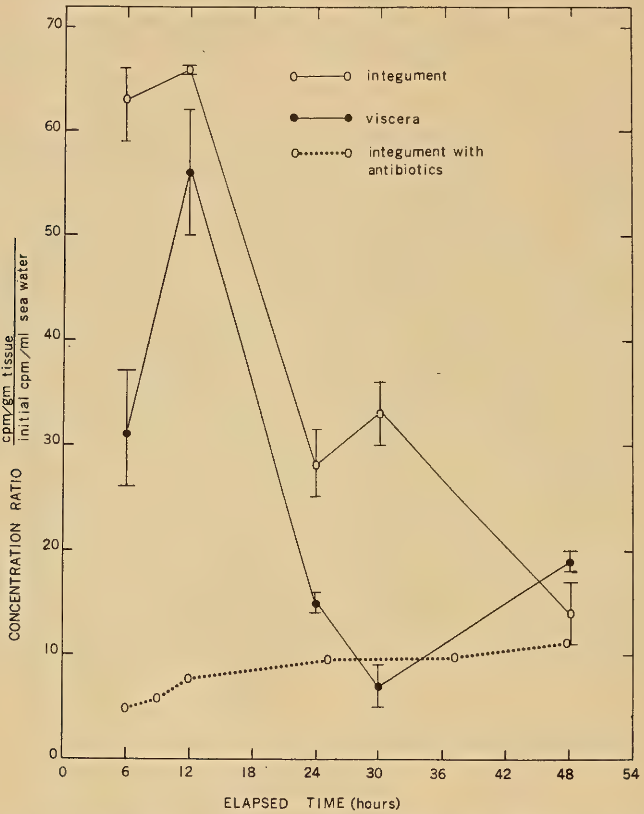

FIGURE 3. The changes in P^{32} activity of the integument and viscera of *H. atra* incubated in filtered sea water enriched with phosphorus, as a function of incubation time. Each point represents the mean from three animals exposed for the same period of time while the vertical lines represent the ranges for each set of values. The P^{32} activity of the integument from animals previously treated with antibiotics is shown for comparison with the activity of the integument and viscera under non-sterile conditions. Each point represented in the antibiotic curve indicates analyses from single animals sacrificed at the times shown.

mals were removed from their containers after 6, 9, 12, 25, 36, and 48 hours. The organs of the viscera were removed and separately analyzed for their P^{32} content. The integument was also subjected to phosphorus analysis. Coelomic fluid was not withdrawn from these sterilized animals. Dehydrating, ashing and counting techniques used for the tissue components of this portion of the study were carried out as before.

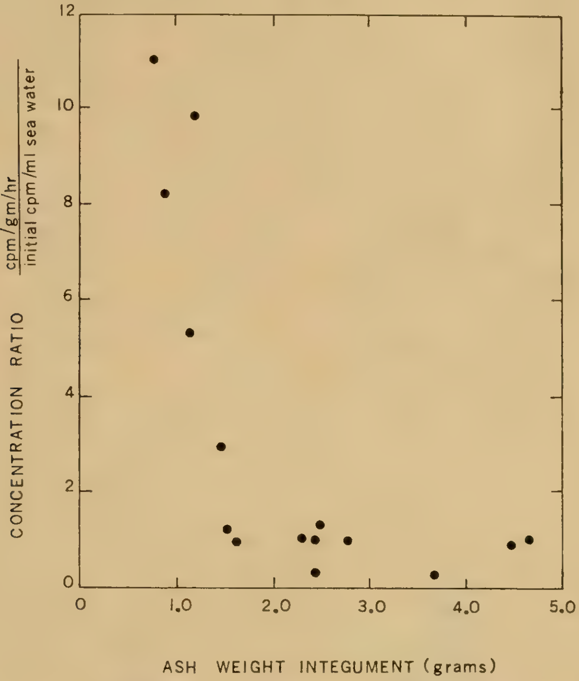

FIGURE 4. The uptake of P^{32} from the enriched sea-water medium by the integument of unrestricted individual specimens of *H. atra*. Each point represents a single organism and the uptake rate is measured in counts per minute of P^{32} activity per gram of integument per hour of incubation time. The incubation intervals were 6, 12, 24, 30, and 48 hours. Animals with ash weights ranging from 0.0 to 2.0 grams were incubated for 6 to 12 hours, while those animals with ash weights between 2.0 and 4.8 grams were incubated for 24, 30, and 48 hours.

RESULTS

The results of the experiments determining the gross removal rate and subsequent distribution of P^{32} to the integument and visceral tissue fractions are shown in Figures 2, 3, and 4. One method for measuring the rate of uptake of P^{32} from solution is by the disappearance of the isotope with time from the ambient medium. A decrease in isotope activity is illustrated in Figure 2 which shows a reduction in medium activity with increasing length of exposure of intact unrestricted specimens of *H. atra*. It can be assumed from this curve that the P^{32} incorporated by the animals is the reverse of the decrease from the solution.

The P^{32} supplied as phosphoric acid that was removed from solution and incorporated in the integument and combined visceral tissue fractions was measured as concentration ratios to allow for variations in animal size and variations in the initial P^{32} activity in the medium. The changes in the concentration ratios of both fractions over the time period indicated are shown in Figure 3. It can be seen that in both fractions there is a rapid initial increase in P^{32} activity between 6 and 12 hours of incubation followed by a rapid decrease in activity in the next 12 hours. The P^{32} content of the integument fraction is consistently somewhat greater than that of the viscera fraction throughout the length of the incubation period even though the fluctuations in both occur at about the same time. The P^{32} content of both tissue fractions after 12 hours of incubation drops sharply but maintains a concentration in excess of that shown by the medium throughout the incubation period. Toward the end of the exposure period the tissues from both fractions begin to approach an equilibrium with the medium with respect to their P^{32} content.

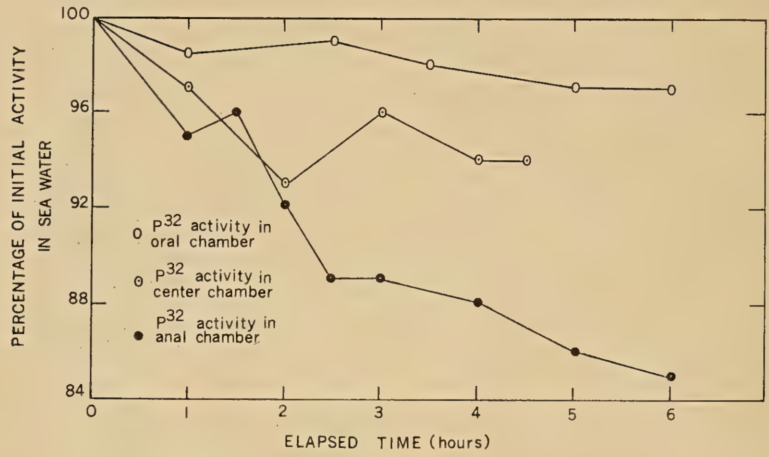

FIGURE 5. Relative decrease in P^{32} activity as a function of time from specified chambers of the split tank using the enriched sea-water medium and individual specimens of *H. atra*. Each curve represents the uptake of isotope by single animals exposed to the phosphorus tracer in one chamber.

The P^{32} concentration ratios of the integuments from unrestricted specimens incubated in the enriched sea-water medium for determination of gross untake are plotted as a function of the total ash weight of the integument. The effect of animal size on the rate of disappearance of the isotope from solution is shown in Figure 4. Correction is made for the length of time that each animal was exposed to the phosphorus tracer. There is a sharp decrease in concentration ratio as the ash weight of the integument increases from 0.8 gram to 1.8 grams. The concentration ratio, however, remains relatively constant in animals with integument ash weight from 2.0 grams to 4.8 grams. By inspection it can be seen that a negative linear regression line could be calculated to fit the values included in the 0.8- to 1.8-gram region on the graph. A second regression line could be drawn through the values in the 2.0- to 4.8-gram region, indicating that animals with integument ash weight greater than 2.0 grams did not concentrate P^{32} from the medium (they have a concentration ratio of about one). Hence it can be assumed that exchange of isotope between this tissue and the solution was occurring in these animals at the time of sacrifice.

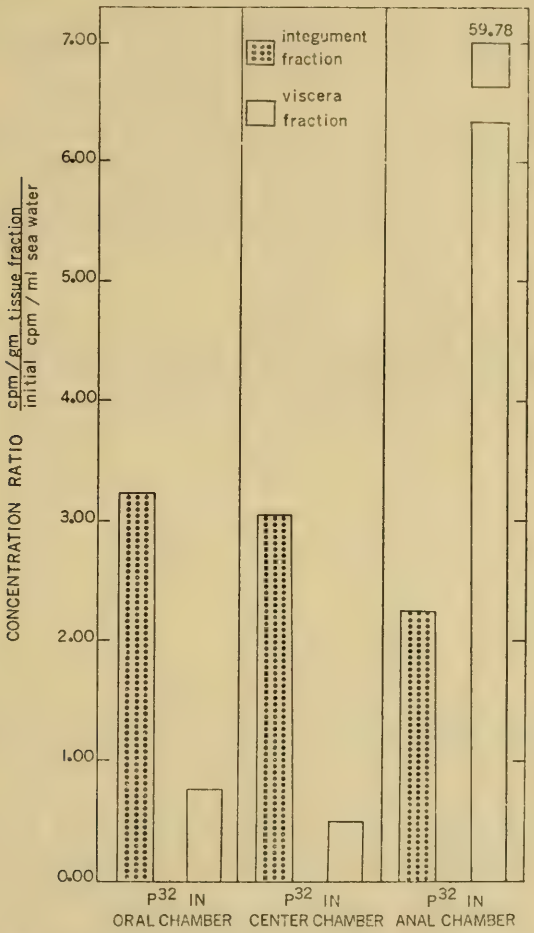

FIGURE 6. The P^{32} concentration rate of the integument and viscera of specimens of *H. atra* exposed to the enriched sea-water medium in the split tank for an incubation time of 6 hours. The concentration ratios of the visceral fraction represent the total combined activities of the gut, left and right respiratory trees and the rete mirabile. The concentration ratios obtained for both the center and anal chambers are average values from two animals, while those values shown for the oral chamber are derived from a single specimen.

The decrease in P^{32} activity in the medium when animals were incubated in the split tank is shown in Figure 5. These are representative activity measurements for individual specimens incubated for a period of 6 hours with P^{32} in the chambers indicated. The P^{32} activity of the medium was found to decrease in all three chambers over the incubation period. The greatest reduction of activity (15% of the initial activity) resulted when the isotope was added to the anal chamber. In contrast, it can be seen that a reduction in the oral chamber of 3% occurred during the same time interval, while the relative decrease of P^{32} activity in the central chamber (where the integument served as the only pathway for uptake) was about 7%. It is noteworthy that very little P^{32} decrease was recorded when the labeled medium was placed in the oral chamber.

A first approximation of the relative efficiency of P^{32} uptake of each of the three pathways into the body can be made by comparing the P^{32} concentration ratios of the integument and the combined total activity of the individual organs of the viscera from experimental animals given the varying isotope exposures. The concentration ratios of the integument and combined viscera of animals incubated in the split tank with the isotope in one of the three chambers are represented in Figure 6. The concentration ratios calculated for the center and anal chambers represent averages from two animals, while those for the oral chamber are from a single animal. The concentration ratios of the integuments of animals in each chamber are consistently uniform, while the concentration of phosphorus in the combined viscera tissue fraction is more variable. Equilibrium had been reached between the viscera and the medium in those animals exposed to P^{32} in the oral and central chambers as indicated by the concentration ratios of approximately one. In contrast, the high concentration ratio of 59.78 in the viscera fraction of animals exposed to the tracer in the anal chamber is indicative of great isotope accumulation.

TABLE I

Percentage of P^{32} activity of various tissue fractions from H. atra incubated in the split tank with radionuclide only in chamber indicated for time intervals not exceeding six hours. Data from the oral chamber were derived from single experiments, while in all the remaining cases averages from two animals under the same conditions were used

Tissue fraction	Chamber with P^{32} activity		
	Oral	Central	Anal
Integument	97.5	96.2	19.0
Viscera: Gut	1.1		11.0
Viscera		1.2*	
Viscera: Respiratory trees + Rete mirabile	1.2		24.0
Coelomic fluid	<0.5	2.6	48.0

* Value represents the combined percentages from gut, respiratory trees, and rete mirabile fractions.

The relative P^{32} distribution in the various organs and body fluids after incubation of animals in the split tank containing the isotope in the different chambers is presented in Table I. When P^{32} was supplied in either the oral or central chamber, the highest per cent of the activity appeared in the integument (97.5% and 96.2%). On the other hand, when P^{32} was supplied in the anal chamber, the respiratory trees and the coelomic fluid contained the greatest percentage of activity. It seems that significant amounts of radioactive phosphorus reach the coelom and the respiratory trees only when the experimental solution is presented in the anal chamber.

Data from the last experiment involving the possible effects of bacterial activities in the observed phosphorus uptake from solution by *H. atra* are presented in Figure 3 and Table II. P^{32} concentration ratios from the integument of unrestricted animals incubated in the enriched sea-water medium (incubation time from 6 hours to 48 hours) after treatment with antibiotics are indicated in Figure 3. From this figure it is apparent that there is a very significant difference in P^{32} accumulation during the first 12 hours of incubation with the isotope, between animals not exposed to antibiotics and those that were treated with antibiotics. At the end of the incubation interval, however, the concentration ratios approximate one another. Instead of rapid P^{32} changes occurring in the integument of the sterilized animals, as seen in this organ of the non-sterilized sea cucumbers, there is a slow but steady concentration of isotope over the 48 hours of incubation.

TABLE II

P^{32} activity of tissue fractions after incubation of antibiotic treated animals in three liters of sea water with added radionuclide. All measurements are in cpm/gram ash tissue fraction

Tissue fraction	Time (hours)				
	6 Hour	9 Hour	12 Hour	25 Hour	36 Hour
Integument	4200	4300	6400	7000	9800
Gut	1400	6200	5200	5800	16900
Right tree	15100	21800	64200	31000	400000
Left tree and rete	13800	34100	30000	64600	251000

The six animals used in the antibiotic experiment were analyzed for the P^{32} activity of their tissue components (Table II). By inspection it is seen that the activity of the integument fraction increases slowly throughout the duration of the experimental period. In a similar manner the gastrointestinal tract fraction also shows a slow but constant increase in radioactivity. The P^{32} activity of the gut component surpasses that of the integument after 25 hours of incubation. The radioactivity within the respiratory trees shows a marked increase with time and is one to two orders of magnitude greater than the activity found in the integument or gut at the same time. The disparity between the uptake figures in Table II for the integument and the respiratory trees may indicate a dissimilar effect of the antibiotics on either the holothurian enzyme systems present in these locations or on the bacterial flora associated with these organs.

DISCUSSION

It has been demonstrated that *H. atra* accumulates the isotope P^{32} from solution and subsequently distributes it to major tissue and organ systems. The uptake data derived from the isolated regions of the test animals served as a comparison to uptake data obtained from phosphorus kinetics of unrestricted animals incubated in larger undivided receptacles.

The disappearance of activity from the enriched sea-water medium in which specimens of *H. atra* were incubated, as shown in Figure 2, is indicative of some active mechanism by which the P^{32} is extracted from solution at a rate faster than it is returned. Approximately 30% of the original P^{32} activity had been removed from the medium after 40 hours of incubation. That the rate of P^{32} uptake is not equal for all regions of the body is readily apparent by examination of Figure 5. The oral portion of the animal was found to remove much smaller quantities of P^{32} from solution and apparently at a constant rate, indicating simple exchange with the medium. One could hypothesize that in nature, little dissolved phosphorus material is obtained by way of the mouth when the animal is engaging in normal feeding activities.

Phosphorus derived from enzymatic reactions at the integumentary surface may contribute significantly to the overall phosphorus content of the integument.

The greater P^{32} uptake and higher rate of accumulation by way of the anal region of *H. atra* is not surprising. Hyman (1955) presents several lines of evidence that indicate the possible use of the respiratory trees in holothurians as a means of ionic and particulate transport. Because there is a continual passage of water to these structures during respiration, a constant exchange with the environment can occur. It can be assumed that the distal portions of these organs are probably constantly exposed to the labeled phosphorus in the medium and that some mechanism, either diffusion or active transport, is responsible for movement across the epithelium of these structures.

The data plotted in Figure 3 show that the rate of P^{32} concentration by sterile specimens of *H. atra* from the enriched sea-water medium is much reduced from that of non-sterile specimens. In view of Rigler's work it can be speculated that the difference in concentration ratios between the sterile and non-sterile specimens of *H. atra* is largely due to epizootic bacterial flora, surface adsorption, or a change in the holothurian's enzyme systems. No data have been presented to exclude the possibility of holothurian phosphorus enzyme inhibition by the antibiotics and this could definitely be a factor responsible for reduction in the observed P^{32} uptake rate in the integument of the sterilized animals. For the present analysis, however, it will be assumed that reduction in isotope uptake in sterilized animals is more due to a decrease in bacterial activity than enzyme suppression. A similar assumption was made by Rigler (1961) when he found that the penicillin-streptomycin antibiotic mixture used to sterilize *D. magna* reduced its bacterial flora to 0.003% of the original count and that the uptake activities of his treated crustaceans were essentially from "sterile" animals.

The sharp rise in the P^{32} concentration ratio of the integument and viscera of animals exposed to the enriched sea-water medium reflects the rapid uptake by surface bacteria (and to a lesser extent uptake by the holothurian) within the first 12 hours. The drop in P^{32} concentration in the tissues could be accounted for by the inhibition of further phosphorus uptake by the bacteria and holothurian due to the buildup of metabolic waste products from both the sea cucumber and bacteria in the test chamber. During this time there is a net release of labeled phosphorus from the holothurian and its bacteria to the medium so that at 24 hours of incubation the absolute amount of P^{32} present in its tissues is less than at 12 hours. The removal rate of phosphorus activity in the medium as shown in Figure 2 verifies the fact that P^{32} is being pumped back into the medium between 12 and 24 hours; that is the time during which the rate of P^{32} disappearance from solution is reduced. The reduction in uptake rate is illustrated by the change in the slope of this graph. This change in slope may indicate that although the bacterial population and organ systems of *H. atra* are still removing P^{32} from the medium, it is being returned to solution after metabolism at a faster rate than it was being returned during the first 12 hours of incubation. The net result is that water samples analyzed for P^{32} activity show a continuing decrease in activity even though the rate of decrease is slower.

Relating the information of P^{32} uptake in non-sterile animals in Figure 3 to the P^{32} uptake activities of the integument and visceral fractions of *H. atra* preincubated in the antibiotic solution (Table II) reveals contrasting results. The slow accumulation of P^{32} activity in the integument as well as the gut fraction of Table II indicates a marked difference in phosphorus uptake kinetics between these sterile animals and the non-sterile animals of Figure 3. The integument and gut fractions of sterile animals demonstrate, in general, the same pattern of P^{32} uptake. If this level of P^{32} concentration is taken as a reference value, then the concentration of phosphorus in excess of this value, such as that of the non-sterile animals in Figure 3, could be attributed to surface bacterial activities. The gradual increase in activities of both the integument and gastrointestinal fractions under sterile conditions can be traced to three possible causes: the uptake may reflect the concentrating activities of the cucumber minus its bacteria, the uptake may be attributed to the gradual repopulation of the animal by bacteria which resume their concentrating activities, or the uptake may result from the diminishing physiological inhibitory effects of the antibiotics on the holothurian phosphorus enzyme systems.

The respiratory trees of sterilized specimens of *H. atra* (Table II) demonstrate an initial amount of P^{32} per gram of tissue that is four times greater than the amount in the integument after only 6 hours of incubation. As the exposure time under sterile conditions progresses, the concentration of P^{32} by the respiratory trees continues to 36 hours when the last tissue sample was taken. At this time the activity per gram of respiratory tree is between 25 and 40 times the activity which is present in the integument. It would appear that the respiratory trees are able to demonstrate a marked P^{32} accumulation under sterile conditions, while the integument and gut show a more modest gradual increase. An explanation for this difference in isotope uptake by the different sterilized tissues would necessarily involve a difference in phosphorus metabolism and ultimate utilization of the radionuclide. Perhaps the respiratory trees are the major site of phosphorus metabolism and transportation in these animals. A comparison of the activity in the respiratory trees from the sterilized animals (Table II) to the concentration ratio attained by the combined visceral fraction of the non-sterile animals exposed to P^{32} in the anal chamber of the split tank (Fig. 6) lends credence to the hypothesis that enzyme inhibition is not a factor in reducing the observed uptake of phosphorus in sterile animals. In both cases the activity present in the respiratory trees is considerable, and largely the result of holothurian activities.

The uptake and concentration of phosphorus in the integument from the enriched sea-water medium seem to be dependent upon the surface area of the animal (Fig. 4) ; that is, as the integument ash weight increases, the concentration ratio decreases. This means that as the mass of the holothurian increases, the amount of surface area per volume exposed to the radioactive medium decreases, as does the quantity of P^{32} per gram that is concentrated in the integument.

This phenomenon may now be further examined in light of what has been demonstrated about the role of bacteria in phosphorus uptake. Since size of holothurians is probably proportional to the biomass of attendant bacteria, it would be expected that a smaller holothurian, having a larger surface area, would also possess a greater bacterial surface area per volume of cucumber than would a larger sea cucumber. This means that the bacteria on a smaller holothurian would concentrate more P^{32} per gram of integument than the bacteria on the integument from a larger animal. Thus, Figure 4 can be viewed as illustrating both bacterial and holothurian surface area in the concentration of P^{32} with the greatest contribution to phosphorus activity coming from the attendant bacteria.

In the analysis of the data from Figure 4 it should be pointed out that the smaller animals (those whose integument ash weights ranged from 0.8 to 1.8 grams) were incubated in the P^{32}-enriched sea-water medium for time periods less than 12 hours. Therefore the concentration of phosphorus by the integument and bacteria was increasing at the time of sacrifice (Fig. 3). The increase in activity is influenced by the surface area of the holothurians within this size range, that is, the concentration ratio decreases as the animals increase in weight (decrease in surface area). The animals exposed to P^{32} for 24, 30, and 48 hours are represented by those individuals with ash weights in excess of 2.0 grams in Figure 4. This group is in equilibrium with the medium since the concentration ratio for these animals is close to one. This equilibrium initiated around 24 hours of incubation appears to be independent of the holothurian and/or bacterial surface area.

After 6 hours of exposure to the radioactive medium in any one chamber of the split tank (Fig. 6), the concentration ratios of the integuments of all the animals tested show a striking similarity. The visceral fractions of animals exposed to P^{32} in the oral and center chamber have concentration ratios of less than one after incubation. Because this tissue showed approximately the same activity as the medium at the time of sacrifice, one is led to the speculation that a steady-state could have been in existence between the viscera and the medium. Probably no real concentration of the phosphorus isotope into the gastrointestinal tract had taken place *via* the mouth or integument during the exposure to the isotope. The low activity in the viscera with P^{32} in the central chamber of the split tank suggests that very little activity had penetrated the integument and become incorporated in the tissues of the visceral fraction. The visceral fraction from animals exposed to the enriched sea-water medium in the anal chamber shows a very high concentration ratio which is indicative of a great deal of P^{32} accumulation. Since it has been shown by Crozier (1916) that the anal sphincter muscle closes off the rectal portion of the digestive tract while water is being brought into the respiratory trees, it is assumed that most of the activity present in this combined visceral fraction is due to phosphorus uptake by these structures and not to uptake or leakage *via* the digestive tract.

A more precise assessment of the P^{32}-concentrating activities of the body portions of *H. atra* can be made from the data of Table I. The contribution of the integument to the total activity in the animal is highest when the oral or central chambers of the split tank contain the radioactive medium. When radioactivity is added to the anal chamber of the tank, the percentage of activity attributable to the integument is decreased. This reduction in the percentage of activity of the integument, however, is due to the marked concentration of P^{32} in the respiratory trees and coelomic fluid. It can be assumed that the P^{32} in this chamber was able to pass into the termini of the respiratory trees and become concentrated there. Since the coelomic fluid fraction of the animals exposed to P^{32} in the central chamber shows such low activity in comparison to the coelomic fluid fraction of those specimens incubated with radioactivity in the anal chamber, it may be said that very little phosphorus had entered the coelom by way of the integument. The high percentage of activity in the coelomic fluid and respiratory trees of the animals incubated with activity in the anal chamber illustrates the probable passage of P^{32} across the membranes of the respiratory trees.

SUMMARY

1. When non-sterile *H. atra* is exposed to a sea-water medium containing P^{32}, a decrease occurs in activity in the medium over time and is greatest during the first 12 hours of incubation. The isotope uptake is reflected by an increase in phosphorus activity in the tissue components.

2. Sterile holothurians show considerably less integumental P^{32} uptake than do non-sterile specimens, indicating active bacterial accumulation of the isotope, and to a lesser degree, uptake by *H. atra.*

3. The removal and concentration of P^{32} into the integument seems to be constant over the entire length of the animal, as well as being a function of the mass of the organ; the larger the integument, the smaller the uptake and concentration of the isotope per gram of tissue.

4. The greatest site of P^{32} uptake from solution appears to be by way of the general integumentary surface and through the anal-respiratory tree complex, while the oral region of the animal removes very little P^{32} from solution.

5. A high accumulation of P^{32} occurs in the respiratory trees under both sterile and non-sterile conditions, indicating a possible metabolic utilization of phosphorus by these organs. The major pathway of phosphorus entry to the coelomic cavity in *H. atra* is by passage across the respiratory trees.

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REFRACTILE BODIES IN THE DEVELOPING AND MATURE SPERMATOZOA OF CHILDIA GROENLANDICA (TURBELLARIA: ACOELA) AND THEIR POSSIBLE SIGNIFICANCE[1]

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The morphology of the mature spermatozoa of acoel flatworms, and their spermiogenesis, are of much interest, in view of the recent suggestions of Franzén (1956a, 1956b, 1958), Ax (1961) and Hendelberg (1965) concerning the possible phylogeny of the group. A detailed study has been conducted in this laboratory for several years, by light and electron microscopy (to be reported elsewhere by D. P. Costello), of spermiogenesis in *Polychoerus carmelensis* and in other members of the Turbellaria Acoela, including *Childia groenlandica,* a typical hermaphroditic marine form. The taxonomy of this species is discussed by Westblad (1945) and Hyman (1959), who consider it to be synonymous with *Childia spinosa,* first described by von Graff (1911) from the Woods Hole region. The morphology of the spermatids and mature spermatozoa of *Childia* has proved to be so remarkable as to prompt an account of the features thus far observed, using, for the most part, light microscopy and cytochemical techniques.

MATERIALS AND METHODS

Living specimens of *Childia groenlandica* were collected from mud dredged from the bottom of Great Harbor, Woods Hole, Mass., through the courtesy of the Marine Biological Laboratory Supply Department. They were maintained in the laboratory, both at Woods Hole and Chapel Hill, in large fingerbowls of sea water changed every 2–3 days, and were kept constantly supplied with cysts and newly hatched nauplii of *Artemia* for food.

Study of living material

The animals were cut into approximately 6 to 8 pieces with sharp, fine needles, on a clean slide; a #1 coverslip was then carefully lowered upon the pieces and very gentle pressure applied, to dissociate the pieces into their constituent cells. These squash preparations were then studied by bright field, dark field, phase and polarization microscopy, at magnifications up to 1500 × (1.8 mm. oil immersion objective). Such preparations remained viable and apparently normal for periods of as much as 10 minutes or longer, but were discarded as soon as they showed any

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signs of drying out. Whole, uncut individuals were also compressed and studied in the living condition.

Photomicrographs (35 mm.) were made of both living and fixed material, in black-and-white and in color, with a Leitz Orthomat camera and compound binocular microscope (through the courtesy of Dr. H. E. Lehman), a Zeiss Photo-Microscope,[2] or an AO compound microscope.

General staining techniques

Permanent slides for light microscopy were prepared. The general procedure followed was to squash the cut-up living worms as described above, but often using two coverslips rather than a slide and a coverslip. After the animals were squashed, the two coverslips (or the slide and single coverslip) were carefully separated and dried, with or without heat, until all traces of moisture had disappeared. All observations were confirmed on smears fixed, after air-drying, by a 30-minute exposure to formalin vapor. [Heat-fixed smears showed good preservation of morphology of the cell-types, as judged by extensive comparisons with living material (compare Plates I and II).] The preparations were then flooded with stain, in the presence of heat, for varying periods of time, washed in distilled water, and heat-dried; they were mounted in Permount or Eukitt.

The stains used were as follows: $\frac{1}{2}\%$ toluidine blue in 1% sodium tetraborate, pH 8.6; 1% aqueous Janus green; 1% aqueous safranin O; Wright's stain; Harris' acid haematoxylin with eosin counterstain; Mallory's triple stain. Various sequential combinations of toluidine blue, safranin and Janus green were used, which yielded information not obtained by using any one stain alone.

Supra vitam staining of living preparations was tested with dilute solutions of neutral red, Nile blue sulfate and Janus green B.

Cytochemical procedures

1. Feulgen method for DNA, with hydrolysis in 1 N HCl at room temperature and at 60° C.; Schiff reagent for 30 minutes; two 3-minute changes of sulfurous acid; running tap water for 5 minutes; dehydration through an ethanol series; counterstaining in 0.5% light green in 95% ethanol; xylene.
2. Saturated Sudan IV in acetone, applied to heat-fixed smears; for demonstration of lipids.
3. Sudan black B for "masked" lipids (Barka and Anderson, 1965).
4. Periodic acid-Schiff and alcian blue (pH 1.7), both separately and in combination, and before and after 1% diastase digestion at 37° C. for 1 hour (Pearse, 1960); for mucopolysaccharides.
5. Methyl green-pyronin (Kurnick, 1955), both dyes being chloroform-extracted before combination; with and without prior RNase (Worthington Biochemical Corp., RAF) digestion (1 mg./ml. in 0.005 *M* phosphate buffer, 37° C. for 1 hour); for RNA.

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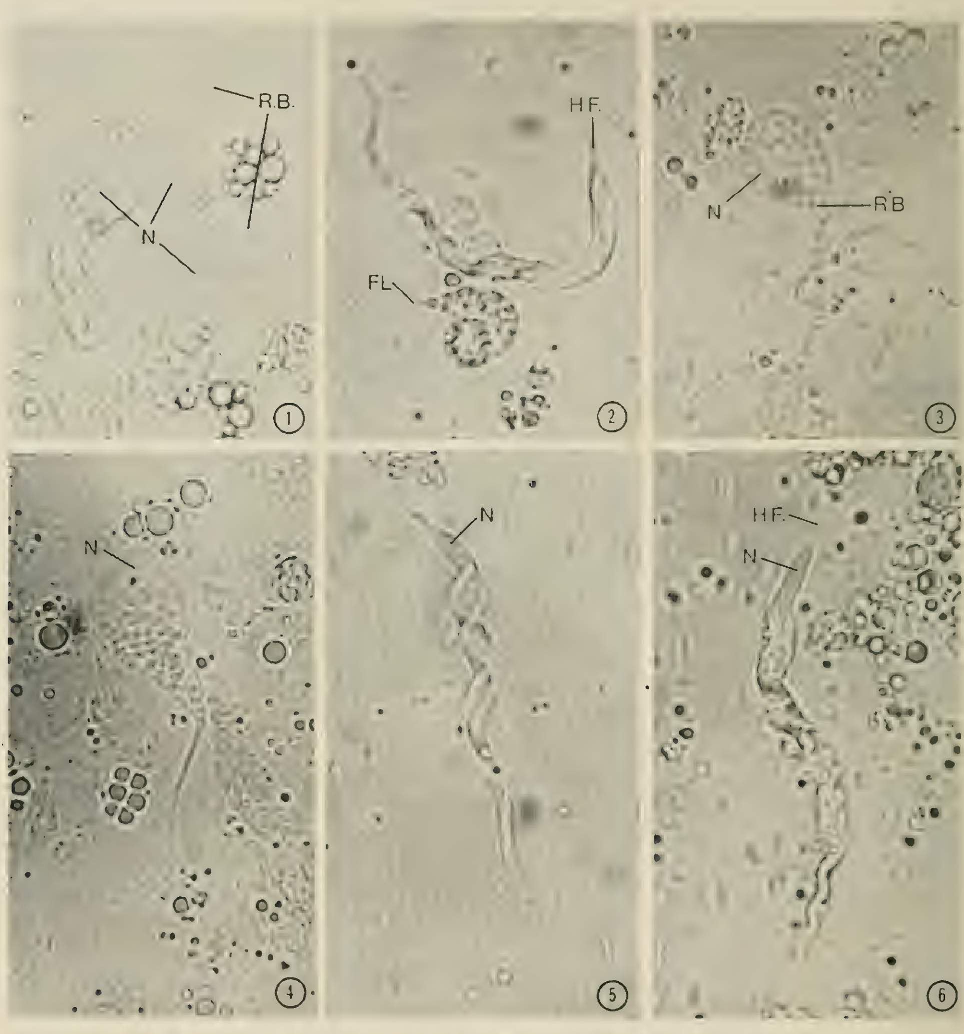
[2] I am indebted to Dr. John N. Couch and Mr. Charles Bland, of the Botany Department, University of North Carolina, for their kind cooperation in allowing the use of their Zeiss Photo-Microscope before we obtained our own.

Abbreviations used in Plates I and II: FL, flagellum; H.F., head filament; N. nucleus; R.B., refractile bodies.

PLATE I



Living unstained spermatids and spermatozoa of *Childia groenlandica*, photographed by bright-field illumination and with a 1.8 mm. oil immersion objective. Magnification of all figures in this plate (after reduction for reproduction): 1305 ×.

Figure 1. Two young spermatids (right) and a very late spermatid (left). In each of the two young spermatids the nucleus is crescent-shaped; in the later spermatid it is not as clearly visible. In all three, the refractile bodies appear as round inclusions in the cytoplasm of the cell. The clusters of dark-rimmed structures elsewhere in the field in this, and succeeding, figures in this plate are fragments of tissue from the *Artemia* used as food for the worms.

Figure 2. A very late spermatid (above) and a younger stage, just beginning the process of flagellar extrusion (below). The loop of the extruded flagellum can be indistinctly

6. Azure B (Swift, 1955), 0.25 mg./ml. in McIlwaine buffer, pH 4.0, 2 hours at 37° C., with and without prior overnight extraction with 10% perchloric acid at 0° C. (Aldridge and Watson, 1963), and with a 1-hour digestion in RNase (1 mg./ml. in 0.005 *M* phosphate buffer) at 37° C., the controls being incubated in buffer alone; for RNA and DNA.

All the above procedures were carried out (with the exception of the Sudan IV method) on both heat-dried smears and on unheated, air-dried smears prepared on albuminized coverslips and fixed in formalin vapor for 30 minutes. Where feasible, confirmation was obtained also by the use of "thick" Araldite sections (see below). In most cases, however, adequate penetration of reagents through this plastic embedding medium could not be obtained.

The following procedures were carried out with smears air-dried, without heat, and fixed by exposure to formalin vapor for 30 minutes:

7. Gomori tests for alkaline and acid phosphatases (Barka and Anderson, 1965). Controls: Incubation without substrate; heat-inactivation (100° C., 1 hour) of enzyme.
8. Simultaneous coupling of azo dyes for alkaline and acid phosphatases (Barka and Anderson, 1965). Controls: As for (7).

Sectioned material

Animals to be sectioned were fixed in ice-cold 4% to 8% cacodylate-buffered glutaraldehyde for 2 hours, thoroughly washed in buffer, and secondarily fixed in osmium tetroxide for 40 minutes; they were then dehydrated in an ice-cold ethanol series, followed by propylene oxide, and embedded in Araldite. Using glass knives, $\frac{1}{4}$- to $\frac{1}{2}$-micron (or thicker) sections were cut on a Porter-Blum MT-1 microtome. Sections were stained, after drying, with toluidine blue and safranin, alone and in combination. Before any of the cytochemical procedures described above were used, the sections were coated with a very thin layer of dilute celloidin or were dried down on albuminized coverslips, to prevent loss of the sections in the reagents.

For electron microscopy, pale gold or silver sections were picked up on uncoated 200-mesh copper grids and stained with uranyl acetate. They were studied and micrographs prepared with a Philips 100 electron microscope, through the courtesy of Dr. M. J. Moses of Duke University, in whose laboratory this portion of the work was done.[3] We are grateful to Dr. Moses for his help and for stimulating discussions.

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[3] The electron micrograph was made by Dr. Donald P. Costello. It is a pleasure to acknowledge my gratitude to Dr. Costello for this assistance, as well as for much additional help and encouragement.

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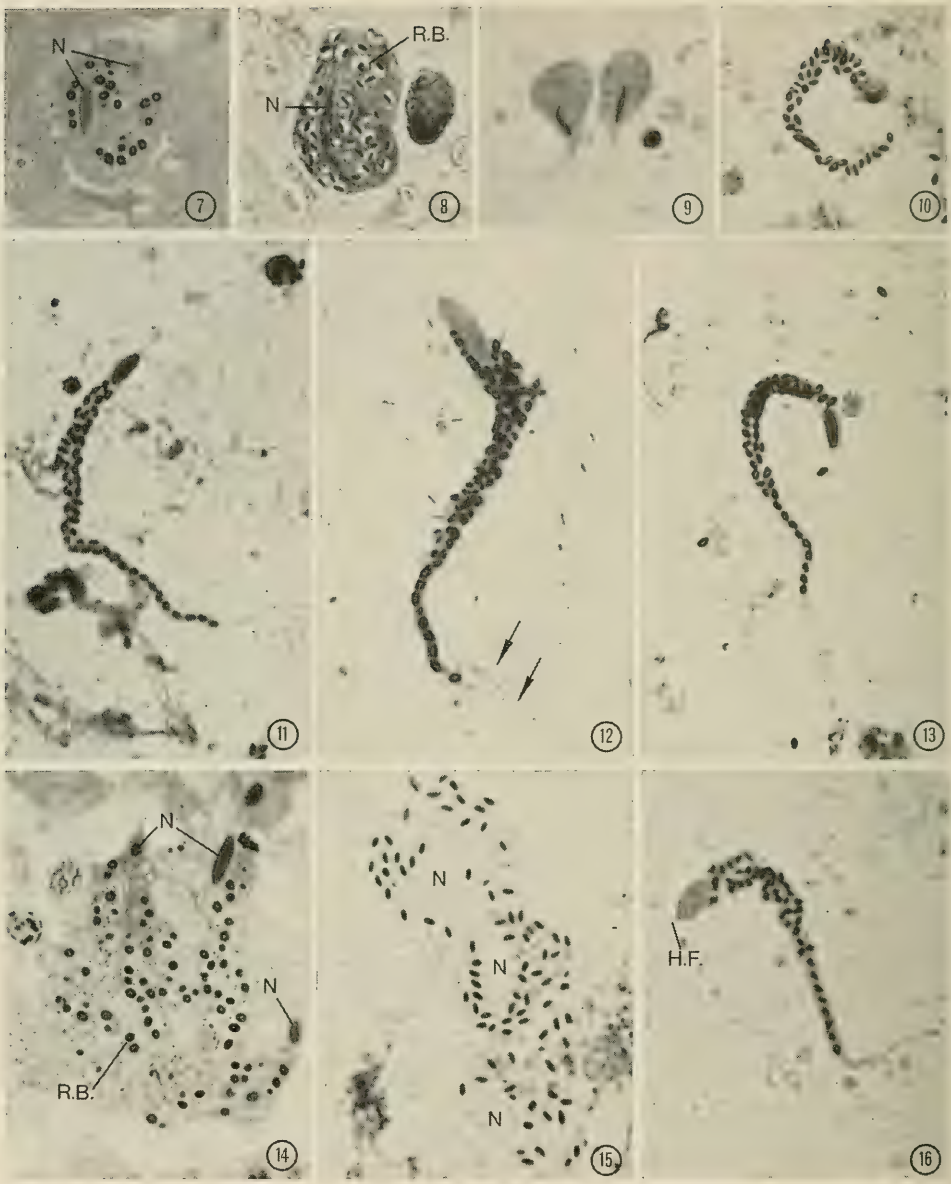
seen in the lower cell, arising from the pointed region near the label and circling back toward the cell. The head filament on the later spermatid appears tenuous because it is out of the plane of focus.

FIGURE 3. Beginning of spermatid elongation.

FIGURE 4. A still later stage of spermatid elongation. The tail is considerably more slender that that seen in Figure 3.

FIGURES 5 and 6. Mature spermatozoa with prominent undulating membranes. In the cell shown in Figure 6, the nucleus is visible, as well as the refractile bodies and the head filament.

PLATE II



Fixed and stained spermatids and mature spermatozoa of *C. groenlandica*, photographed by bright-field illumination.

FIGURE 7. Young spermatid with portions of two nuclei; the crescent-shape of the unsectioned nuclei seen in the preceding figures is only suggested here, because only a portion of each of the nuclei is included in the plane of section. The refractile bodies are darkly stained. Glutaraldehyde fixation with post-osmication; $\frac{1}{2}$-micron Araldite section; safranin. Magnification: 1305 ×.

RESULTS

Mature spermatozoa (Figs. 5, 6, 11–13, 17)

The living spermatozoon of *Childia* has several conspicuous features, all of which are also demonstrable in stained material (compare Figs. 5 and 6 with Figs. 11–13 and 17). The nucleus is anterior in position, about 5 microns long and oval in shape; it is somewhat greenish in color. Projecting anteriorly from the center of the spermatozoon's head (Figs. 6, 11) there is frequently, but not always, a long slender filament which varies in length from one-half to more than twice the length of the nucleus. This variability may be due to the fragile nature of the filament and to the fact that it could easily become broken off when a coverslip is added to the preparation. Careful study of sectioned spermatozoa in the intact body of the animal has thus far revealed no evidence of this head filament; possibly it represents an acrosome reaction elicited by contact of the spermatozoon's head with the glass of slide and/or coverslip. Such a reaction is known to occur on occasion with the sperm of many other marine forms (Dan, 1967). The filament is too thick to be a flagellum of the usual type, and it was not seen to move in living preparations.

Posterior to the nucleus, there is a double row of highly refractile bodies which, in the living condition, are brilliantly greenish in color. They are somewhat ovoid in shape and are arranged in an orderly fashion along almost the entire length of

FIGURE 8. Young spermatid at approximately the same stage as that shown in Figure 7, but with only one nucleus present. The bipartite nature of the refractile bodies is clearly evident, and there are at least two light areas in the nucleus. Heat-fixed smear; Janus green and safranin. Magnification: 1305 ×.

FIGURE 9. Two spermatids beginning elongation. Note the sharply defined appearance of the nuclei, as compared with those shown in Figures 7 and 8; the regions occupied by the refractile bodies appear as clear light areas in the cytoplasm. Heat-fixed smear; Feulgen and light green. Magnification: 618 ×.

FIGURE 10. A somewhat more advanced spermatid. The refractile bodies have now assumed their definitive arrangement in pairs along the length of the tail, but the nucleus is still more or less crescent-shaped. Heat-fixed smear; safranin. Magnification: 1000 ×.

FIGURE 11. Mature spermatozoon, with rather slender oval nucleus, conspicuous long head filament and heavily stained refractile bodies. Heat-fixed smear; safranin; Magnification: 1000 ×.

FIGURE 12. Mature spermatozoon, with somewhat atypical configuration of refractile bodies extending up into the nuclear area. At the posterior end, the undulating membrane is flattened and spread, and two dark peripheral lines can be seen (arrows). Heat-fixed smear; toluidine blue-safranin. Magnification: 1305 ×.

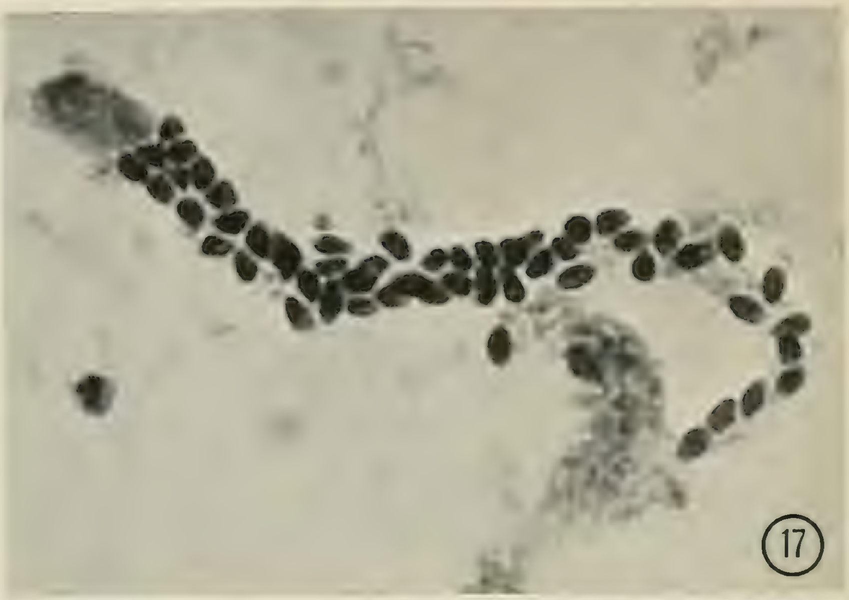
FIGURE 13. Mature spermatozoon. Heat-fixed smear; safranin. Magnification: 1000 ×.

FIGURE 14. Group of advanced spermatids and/or mature spermatozoa. Portions of at least three, and possibly four, nuclei are visible, as well as the characteristic linear arrangement of the refractile bodies. Glutaraldehyde fixation with post-osmication; $\frac{1}{2}$-micron Araldite section; safranin. Magnification: 1305 ×.

FIGURE 15. Young spermatids stained for alkaline phosphatase. The refractile bodies show a strong positive reaction for presence of the enzyme, but there is no deposition of precipitate in or on the nuclei, which appear merely as clear areas. Formalin vapor fixation; Gomori method for alkaline phosphatase. Magnification: 985 ×.

FIGURE 16. Late spermatid, from the same slide as the younger stages shown in Figure 15. In this older cell the nucleus (or the perinuclear region) is faintly stained, and there is a somewhat darker rim at the apex. Head filament extrusion had apparently just begun when the spermatid was fixed. Formalin vapor fixation; Gomori method for alkaline phosphatase. Magnification: 985 ×.

PLATE III



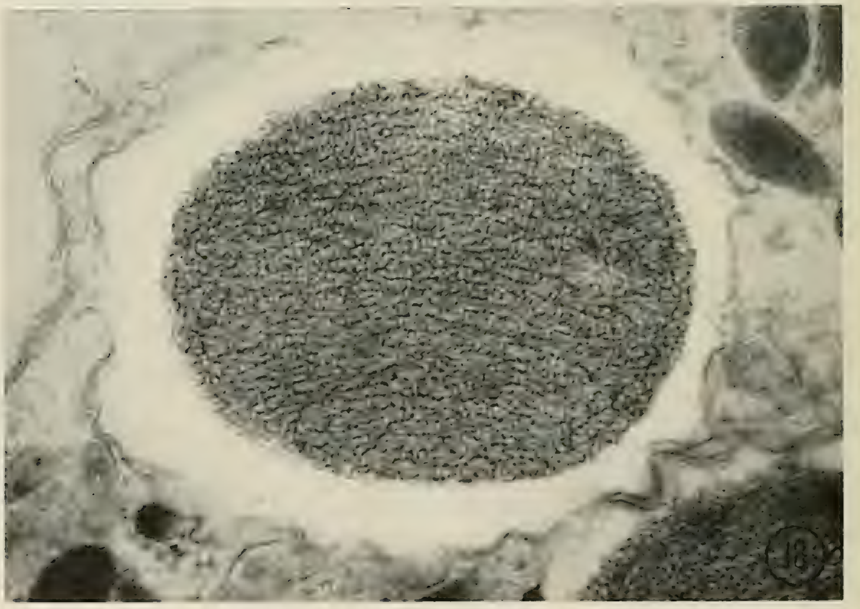


FIGURE 17. Very late spermatid. Note the bipartite staining qualities of the refractile bodies, which in the most posterior region can be clearly seen to have a small dark core in a somewhat less intensely stained matrix. Heat-fixed smear; Janus green. Magnification: 3200 ×.

FIGURE 18. Electron micrograph of single refractile body from a spermatid. There is a prominent ruffled membrane, with a non-electron-dense region beneath, of variable thickness

the spermatozoon. Where the tail begins to taper narrowly at its terminus, the double row is reduced to a single row, which gradually disappears at the tip of the tail. All the refractile bodies are approximately the same size (*ca.* 1.5 microns in their longest axis); their number is quite variable, from 43 to 72 being counted in a random sample of 25 stained intact spermatozoa in smear preparations.

Probably the most obvious single feature of the living mature spermatozoon is the undulating membrane. It is composed of relatively clear, somewhat glassy-appearing cytoplasm, and the cell superficially resembles a trypanosome. There is evidence (to be reported elsewhere) from electron micrographs that two axial filament complexes are incorporated into this undulating membrane; an indication of this can be seen in Figure 12, on the light microscopic level, at the extreme distal end (arrows). Movement of the living sperm is usually quite pronounced and there are rapid undulations which proceed anteriorly along the length of the membrane from the tip of the tail.

In mature spermatozoa stained with Janus green, toluidine blue, safranin, Mallory's triple stain or Wright's stain, both the nucleus and the refractile bodies along the tail are intensely stained (Figs. 11–13, 17) and the cytoplasm is very lightly colored. After use of the Feulgen method, only the nucleus gives a positive reaction, the refractile bodies now resembling vacuoles which are pale in their centers and rather intensely stained with light green at their peripheries. In some Feulgen preparations, there is a suggestion, only, of a very slightly darker or pinkish structure at the center of each "vacuole." The nucleus in Feulgen-stained spermatozoa is considerably more slender than that observed in similar sperm stained by other methods; this suggests that there may be an acrosome, basophilic but Feulgen-negative, overlying the nucleus. In preparations stained with Harris' acid haematoxylin and eosin, the nucleus stains deeply but, surprisingly, the areas occupied by the refractile bodies again appear as clear areas in the pink substance of the eosin-positive tail cytoplasm, just as they do after Feulgen treatment. This lack of staining with haematoxylin probably explains why the refractile bodies were not reported by earlier observers, most of whom stained their preparations with haematoxylin.

A further indication of the different chemical nature of the nucleus, as opposed to the refractile bodies, is afforded by phase contrast and dark-field observations, where it is found that the nucleus appears dark and the refractile bodies stand out brilliantly light in contrast. Study of both living and fixed and stained mature spermatozoa (and earlier stages) with the polarization microscope and a 1.8 mm. oil immersion objective revealed a slight degree of birefringence in some, but not all, granules, particularly when a first-order red plate was used. The probable basis for this finding will be discussed below, in connection with the fine structure of the refractile bodies.

There is no immediate uptake (over a period of 30 minutes) *in vivo* of dilute neutral red by the refractile bodies in either spermatids or mature spermatozoa. As a preparation aged, however, occasionally one or two refractile bodies in a given cell were quite intensely stained at their peripheries. The significance of

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but averaging about 0.15 micron. The main central portion of the refractile body is composed of lamellae which are sectioned longitudinally. The dark bodies at upper right and lower left are dense bodies, of uncertain significance. Glutaraldehyde fixation with post-osmication; uranyl acetate stain. Magnification: 64,000 ×.

this observation is not clear; it may very well represent nothing more than a change in the permeability of the refractile body's surrounding membrane, as a consequence of injury or impending death. No matter how long the preparation was allowed to stand, intense neutral red staining of all or most of the refractile bodies after death did not occur. This is in contrast to the results obtained after Janus green *supra vitam* staining, when no uptake of the dye occurred in the refractile bodies while the cells were alive, but intense staining was sometimes observed after their death (as judged by age of the preparation and loss of motility in cells known to move under more favorable circumstances). Nile blue sulfate did not stain the refractile granules discernibly, no matter how long it was left on.

Spermatid stages

The refractile bodies can be found and readily identified in very young spermatids and, under favorable conditions, in spermatocytes as well; this offers a useful marker in both living (Figs. 1–4) and fixed and stained (Figs. 7–10, 15, 16) material.

During the maturation divisions occurring in the spermatocyte stages, there are usually four or more nuclei within an undivided or partially divided cytoplasmic mass; this delay in cytokinesis persists for a variable period during the early spermatid stages (Fig. 7). In the youngest spermatids observed to have individual cell boundaries, the nuclei are somewhat crescent-shaped and rather broad, as seen in the living condition (Fig. 1) and in fixed and stained preparations (Figs. 7, 8, 15). In Feulgen-stained material, the chromatin is sharply defined in a slender, sickle-shaped nucleus; this reflects the difference noted above between spermatozoa stained with the usual routine dyes and those stained by the Feulgen technique. Frequently, one or more round colorless areas are present in the spermatid nucleus; these are presumably not nucleoli, because although they are Feulgen-negative, they fail to stain with azure B or pyronin.

The refractile bodies appear at least as early as the secondary spermatocyte stage, and may be present as far back as the primary spermatocyte stage. Usually there are only one or two present at first in spermatocytes, but their number increases rather rapidly; the minimum number counted with certainty in smears of the youngest spermatids is 7, and usually there are considerably more. This increase in number continues with growth of the young spermatid, and in older cells of this type, 20–30 refractile bodies are present. In the elongating spermatid (Fig. 10), 40–50 can usually be counted, as compared with as many as 70 or more in the mature spermatozoon.

From its initial spherical shape (Fig. 1), the young spermatid gradually elongates; the very late spermatid (Figs. 1, 2, 16) is similar in appearance to the mature spermatozoon except that its nucleus is broader. With continuing growth and elongation of the late spermatid, the nucleus gradually assumes the slender oval shape characteristic of the mature spermatozoon, and the granules become aligned in the definitive double row along the tail.

Cytochemistry of the refractile bodies in spermatids and mature spermatozoa

Table I summarizes the observations made on the refractile bodies following all the staining and cytochemical procedures tested, under the conditions stipulated in

"Materials and Methods." No qualitative or quantitative differences were found between the refractile bodies of spermatids and those of mature spermatozoa by light microscopy, except for the changes in their numbers discussed above.

Taken together all the findings suggest that these bodies have a complex structure which is probably at least bipartite in nature, and may very well include more than two components. A central region containing one or more acid mucopolysaccharides and a peripheral region characterized by the presence of neutral or basic polysaccharides are clearly evident. The cytochemical tests for phosphatases are much more equivocal: that for alkaline phosphatases, as determined by the Gomori method (Figs. 15 and 16), appeared definitely positive but when the azo-simultaneous coupling method was used, there was a suggestion, only, of a positive reaction, and that occurred at the centers of the refractile bodies. Similarly, the acid phosphatase determination by the Gomori method yielded a questionable posi-

TABLE I

Summary of observed appearance of refractile bodies in developing and mature spermatozoa of C. groenlandica

Test	Appearance of refractile bodies
Toluidine blue, pH 8.6	Deep purplish blue
Safranin O	Intense red
Janus green (fixed material)	Blue, sometimes with darker center
Harris' acid haematoxylin	No discernible staining
Eosin	No discernible staining
Wright's stain	Deep blue
Mallory's triple stain	Bright blue
Light green	Slight accumulation at peripheries
Cytochemical tests:	
Feulgen	No staining except for very questionable pinkish center; DNA-negative
Methyl green-pyronin	Intense red
Methyl green-pyronin after RNase	Intense red; RNA-negative
Azure B, pH 4.0	Deep purplish blue; DNA-negative
Azure B, pH 4.0 after RNase	Deep purplish blue; RNA-negative
Azure B, pH 4.0 after PCA	Deep purplish blue; RNA-negative
PAS	Pink peripheries; colorless or nearly colorless centers; mucopolysaccharide-positive at peripheries
PAS after diastase	As for PAS; glycogen-negative
Alcian blue, pH 1.7	Small central blue granule in clear area; acid mucopolysaccharide-positive at centers
Alcian blue/PAS	Pink peripheries; blue centers
Gomori for acid phosphatases	*Possible* dark peripheries, clear centers; questionable acid phosphatase-positive at peripheries
Gomori for alkaline phosphatases	Intense black; alkaline phosphatase-positive
Azo-simultaneous coupling for alkaline phosphatases	Probable small dark red areas in centers; greenish peripheries; questionable alkaline phosphatase-positive
Azo-simultaneous coupling for acid phosphatases	No discernible staining; acid phosphatase-negative
Sudan IV, Sudan black B	No discernible staining; lipid-negative
Vital stains: Neutral red, Nile blue sulfate, Janus green	No discernible staining

tive reaction at the peripheries, but no reaction in the centers of the refractile bodies, and the same was true after use of the azo technique. At the present time it is not possible to draw any firm conclusions concerning the presence of phosphatases; a more valid approach will be through a combination of cytochemistry and electron microscopy, and such tests will be done in the near future.

The persistence of staining with methyl green-pyronin and with azure B after RNase and perchloric acid extraction rules out the possibility that RNA is present in significant amounts. Appreciable quantities of DNA are likewise absent from the refractile bodies.

Fine structure of the refractile bodies

There is a definite orientation of lamellae in the central region of the refractile body as seen by electron microscopy; the example shown in Figure 18 exhibits a longitudinal arrangement of somewhat irregular layers, but refractile bodies sectioned in other planes show all gradations between this arrangement and a polygonal one seen if the plane of section happens to be exactly transverse across the refractile body. As noted above, some refractile bodies show birefringence when viewed with the polarization microscope and others do not; this implies that orientation of the bodies in the tail of the spermatozoon is random, so that the plane of section will also be a matter of chance. A ruffled membrane surrounds the refractile body and is separated from it by a space variable in width but averaging about 0.15 micron.

Mitochondria of typical appearance but varying abundance (depending on the stage of the developing spermatozoon) are present; there appears to be no particular pattern to the spatial arrangement of these organelles, with reference to the refractile bodies.

Discussion

The possible significance of the refractile bodies

Spermatozoa are notable for the absence of any "excess baggage," so it is likely that the presence of large inclusions such as the refractile bodies has functional significance. There are at least two lines of speculation about this possible significance.

(1) The morphology of the refractile bodies, as viewed by electron microscopy (Fig. 18), is strikingly similar to that of the granules in basophilic leucocytes and mast cells (Fedorko and Hirsch, 1965; Ginsburg and Lagunoff, 1967) and even to eosinophil leucocyte granules at an earlier stage of their development (Fawcett, 1966; Fig. 115). In all three, there is an orientation of lamellae in one plane (or more; see Winqvist, 1963), with a ruffled membrane at the periphery (compare Fig. 18 in the present paper with Figs. 112 and 115 in Fawcett, 1966). The cytochemical reactions of leucocyte granules and refractile bodies are likewise similar in many ways. For example, it is well known that the granules of mature eosinophils and of mast cells (which appear to be closely related to basophils in structure and function) are among the few cases known of material which gives a positive reaction with methyl green-pyronin tests for RNA, but is resistant to extraction by RNase or perchloric acid (Pearse, 1960; Barka and Anderson, 1965). The refractile bodies share this property. Both basophil leucocyte granules and refractile bodies

stain intensely with the methylene blue component of Wright's stain and with toluidine blue, and both fail to stain with eosin. Another cytochemical resemblance among mast cell and basophil leucocyte granules, and the refractile bodies, is the demonstrated presence in all three (by very different methods) of a sulfated acid mucopolysacccharide. In the basophil leucocyte and mast cell granules, this component has been identified as heparin (Ackerman, 1963; Spicer, 1963). It will be instructive further to characterize the mucopolysaccharide in the refractile bodies, in order to determine if it, too, is heparin or a heparin-like compound.

The granules of eosinophils (but not those of basophils) are generally conceded to be lysosomes, on the bases of their observed lytic action (Hirsch, 1962; Archer and Hirsch, 1963) and cytochemical properties (Bainton and Farquhar, 1966; Fawcett, 1966). The fine structure of the refractile bodies and of basophil leucocyte granules suggests that they might represent a more primitive stage of a hypothetical generalized enzyme-storage organelle than do the eosinophil's granules, with their peculiar central crystalloid structure.

The possible significance of lysosomes with regard to the refractile bodies of the spermatozoon of *Childia* and to the fertilization of the egg is suggested by the cortical layer reaction known to occur in such forms as the sea urchin egg (see Endo, 1961). Fertilization of the egg results in an immediate "explosion" of the cortical granules,[4] so that they burst open and release their contents into the perivitelline space. This process apparently results in hardening of the fertilization membrane, so that entry of supernumerary spermatozoa is prevented; de Duve and Wattiaux (1966) have suggested this as a possible function for lysosomes. Dalcq (1965) has demonstrated the presence of one or more acid phosphatases in the cortical granules of the egg of *Paracentrotus,* implying that these could be classified as lysosomes.

The cortical granule-release phenomenon, in those forms which possess such granules, may be represented in *Childia* by a *sperm cell's* contribution of analogous structures, in the form of the refractile bodies. Although the cytochemical reactions of the refractile bodies apparently do not support the idea of their inclusion in the category of lysosomes, as presently defined (de Duve and Wattiaux, 1966), there is the possibility, as those authors point out in the same paper, that (p. 480) ". . . the primitive lysosome which we postulate may have been very different from what it is today and may have included neutral and alkaline as well as acid hydrolases; separation of these enzymes into distinct groups may have occurred subsequently in evolution."

(2) Acoel gametes have two unique features: (a) The eggs mature, undergo penetration by the spermatozoa, go through the maturation divisions to produce internal polar bodies, and proceed to the metaphase of the first cleavage division within the bodies of the hermaphroditic worms; at this stage they come to rest. The block to development present in this resting first cleavage metaphase condition is not released until after the eggs are laid and enter sea water. (b) Acoel sperm also have a unique feature, the presence of varying numbers of refractile bodies. Perhaps these two unique features are related to each other. The evidence on the nature of the refractile bodies presented here is compatible with the idea of their

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[4] This rapid "explosion" is very reminiscent of the behavior of phagocytosing leucocyte granules, as documented with cinephotomicrography by Hirsch (1962, *et seq.*).

playing a role as source of an activation substance, which could be released into the egg cytoplasm during the period after entrance of the spermatozoon. It is not yet known whether the entire spermatozoon enters the egg of *Childia* at fertilization; probably it does, as judged on the basis of known facts about closely related forms (*Polychoerus,* for example; see Costello, 1961).

Some comparative aspects of the morphology of spermatozoa

Von Graff (1911) and Westblad (1945) reported that the mature spermatozoon of *C. groenlandica* is 50–60 microns long; in the same paper Westblad described the presence of an undulating posterior region in the sperm cells of this form. Neither worker mentions the presence of any refractile bodies or granules. An undulating membrane has long been known to be characteristic of the spermatozoa of certain amphibians, some fish, and some Turbellaria, according to Wilson (1925). Notable among the Turbellaria are the spermatozoa of *Macrorhynchus, Darwinia* and *Aphanostoma* (Koltzoff, 1909) and *Haploposthia* (Westblad, 1945).

Conspicuous inclusions within spermatozoa appear to be relatively uncommon. Retzius (1906) described granules in the mature male gametes of two unidentified turbellarians and of the gastropod, *Buccinum.* Other descriptions of granules of comparable size in spermatozoa are given by Repiachoff (1893), for another unidentified turbellarian, and Luther (1912) for the acoel *Palmenia tvaerminnensis.* In a more recent paper, Jamuar (1966) reported the presence of "mitochondrion-like bodies" in electron micrographs of the mature spermatozoa of the nematode *Nippostrongylus brasiliensis.* These inclusions are membrane-bounded and have a crystalloid substructure; they are arranged in linear fashion in the non-nucleated portion of the sperm cell. Mitochondria of the usual appearance are also present, interspersed among the mitochondrionlike bodies, which were found to be DNA-negative, RNA-negative, and PAS-positive. Clark *et al.* (1968) have described, in abstract form, the occurrence of what they interpret to be proacrosomal vesicles in electron micrographs of the spermatozoa of *Ascaris lumbricoides* var. *suum.*

The morphology of the refractile bodies in spermatids and spermatozoa of *Childia,* as seen by electron microscopy, is very reminiscent of that reported by Silveira (1967) for an acoel turbellarian identified as *Anaperus gardineri.* However, her findings are not in accord with preliminary studies we have made of spermiogenesis in *Anaperus* by electron microscopy (Costello and Henley, unpublished data); instead, Silveira's results closely resemble those reported here, for *Childia.* The refractile bodies in our *Anaperus* material do not have the striking outer membrane, nor the oriented substructure she describes and figures, but are much more like similar refractile bodies in the spermatozoa of *Polychoerus* in being smaller, lacking the conspicuous membrane and having no discernible substructure. It is possible that Silveira was actually working with *Childia* and not with *Anaperus* (the two are superficially quite similar in appearance, and they often are found in the same general collecting area in Great Harbor at Woods Hole). Even this resolution of the difference in results meets with a difficulty in her statement (p. 98) that ". . . as far as we have observed, the granules are not included into the mature sperm cells." The present report documents quite clearly the fact that the refractile bodies (= "granules") are definitely present in all stages of spermiogenesis, including the mature spermatozoon, at least until the time of mating.

Very recently, Longfellow (personal communication) has found that in the mature spermatozoon of the rock barnacle *Balanus*, there is a linear arrangement of from 4 to 12 spherical structures, which stain intensely with toluidine blue, and by electron microscopy are seen to be quite dense but with no apparent substructure. He has also observed, by electron microscopy, a long, core-like structure extending through much of the length of the spermatozoon; this may be the nucleus.

It seems quite likely that as the spermatozoa of more and more forms are examined with electron microscopy, other examples of such inclusions will be found. Perhaps further clues will then be available for clarifying the enigmatic role of such structures as the refractile bodies in the spermatozoon of *Childia*.

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SUMMARY

1. Living and fixed mature spermatozoa of *Childia groenlandica* (Turbellaria: Acoela) have conspicuous refractile bodies present behind the oval nucleus, in a double longitudinal row in the tail; an undulating membrane is also evident, as well as (in some cases) a slender head filament which is not a flagellum but which may be the result of an acrosome reaction. The living spermatozoa are 50–60 microns long and moderately motile. In living and fixed specimens, the refractile bodies are found at least as early as the secondary spermatocyte stage and increase in numbers rapidly during the spermatid stages.

2. The following results were obtained with cytochemical tests of the refractile bodies: (a) Feulgen, for DNA, negative; (b) methyl green-pyronin and azure B, for RNA, positive before and after RNase and PCA extraction; (c) alcian blue/PAS for polysaccharides, positive before and after diastase digestion, for small accumulations of acid mucopolysaccharide(s) in centers, only, of refractile bodies; (d) Sudan IV and Sudan black B, for lipids, negative; (e) Gomori, for alkaline phosphatases, positive; (f) azo dye-simultaneous coupling, for alkaline phosphatases, questionably positive in small central area; (g) Gomori, for acid phosphatases, very questionably positive at peripheries; (h) azo dye-simultaneous coupling, for acid phosphatases, negative. The refractile bodies stained heavily after toluidine blue, safranin, Janus green, Wright's stain and Mallory's triple stain; they did not stain after Harris' acid haematoxylin, eosin or light green, nor (*supra vitam*) with neutral red, Nile blue sulfate or Janus green B.

3. By electron microscopy, the refractile bodies were found to have an electron-dense oriented substructure of definite lamellae in the central region; this is surrounded by a ruffled membrane which is separated from the central region by a variable distance averaging approximately 0.15 micron. The fine structure of the refractile bodies is strikingly reminiscent of that of certain other cell inclusions in other forms, most notably the granules of basophil leucocytes and mast cells. This resemblance is reflected, to some extent, in their cytochemical characteristics.

4. The hypothetical significance of the refractile bodies is discussed in relation to the possibilities that they may (a) play a lysosome-like role in fertilization of the egg, or (b) remove the block to development which occurs after fertilization and during the first cleavage metaphase until the eggs are laid in sea water.

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COPEPOD PHOTOTAXIS AND VERTICAL MIGRATION INFLUENCED BY XANTHENE DYES[1]

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Vertical migration of pelagic copepods has been extensively investigated but general conclusions concerning the factors responsible have not been reached. One reason is the diversity of response to environmental factors in different species. Another is the complexity of the responses to light believed to be involved. The same species may be photopositive or photonegative depending upon light intensity (Johnson, 1938), temperature (Parker, 1902), and whether the light is from a point source or diffuse (Schallek, 1943). Further, copepods in different stages of development may show different reactions (Conover, 1956). Finally, diurnal migration may exhibit an endogenous rhythm (Harris, 1963).

This paper reports an investigation of phototaxis in three of the most common copepod species in coastal waters of Brazil. Three aspects were examined: (1) reactions to a point source of light; (2) the vertical migratory cycle in diffuse light in the laboratory as compared with vertical distribution in natural waters; and (3) the presence of an endogenous rhythm of vertical migration. The copepod species chosen for the experiments were *Calanopia americana,* known to migrate very markedly in close correspondence with the variation of daylight (Clarke, 1934); *Acartia lilljeborgi* in which the adults migrate markedly but the young do not; and *Paracalanus crassirostris* which migrates only slightly.

It was noticed that a xanthene dye altered phototaxis in copepods. In investigating this further, the xanthenes rhodamine B, fluorescein sodium, and pyronine B have been studied with respect to their action on phototaxis, on vertical migratory cycles as observed in the laboratory, and on locomotion. The results reported show that the three species of copepods differ in their responses to light and that rhodamine B brings about other differences in behavior between species.

METHODS

The copepods were caught with a plankton net from the pier of the Marine Station of the Instituto Oceanográfico at Ubatuba, Brazil. They were used in experiments soon after capture or within 18 hours at the latest.

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[2] The research was carried out during the tenure of a Visiting Professorship supported by the Organization of American States. Present address: Department of Zoology, Duke University, Durham, North Carolina, U.S.A.

Responses to a point source of light

Copepods were placed in observation chambers on the stage of a dissecting microscope and illuminated horizontally by a 6-volt Leitz microscope lamp focused to give maximum field width. The distance from the lamp housing to the center of the stage of the microscope was 18.3 cm. The intensity of the light at the forward edge of the lamp was 3129 lux. A water heat filter of 6.5 cm. path length and large volume was used.

The observation chambers were rectangular plastic dishes 53 mm. × 35 mm. × 15 mm., covered on the sides with black paper. An aperture 10 mm. × 35 mm. in the black paper permitted light to enter one end. A transverse line on the bottom of the chamber and at right angles to the beam of light served as a reference mark in recording phototactic movements (see below). The experiments were carried out in a darkened room, and the observation chamber was screened from stray light by a black enclosure which contained the microscope and had apertures for the oculars and for the entering beam of light.

The following procedure was employed in making observations. Three chambers (described above) A, B, and C containing similar numbers of copepods in 8 or 12 ml. of sea water were placed in the dark to insure random distribution of the animals. Chamber A was then arranged on the stage of a dissecting microscope. At a signal, the animals were suddenly exposed to a beam of light entering the end of the chamber. The number of animals exhibiting positive phototaxis, negative phototaxis or non-phototactic somersaulting was recorded during each 10-second period for three minutes. The somersaulting was a continuous rotational swimming about a horizontal axis. Chamber A was then placed in the dark and chambers B, then C, were successively arranged and similarly observed. The series of observations on the three chambers were immediately repeated once or twice. Then 2 or 3 ml. of rhodamine B, fluorescein sodium, or pyronine B solution were added to two chambers and a similar volume of sea water was added to the third. The activity of the animals was again recorded in successive trials for about 30 minutes. The effects were observed to be maximal within this interval. In order to observe reversibility, the compounds were replaced by sea water and countings were continued for one or more trials.

For each 3-minute trial the total number of animals moving across the field and the number showing photopositive or other reactions were determined. The percentage of photopositive reactions was calculated for each three-minute trial. The mean percentage of photopositive reactions was also calculated for each series of trials in each experiment.

Migration experiments

Vertical migration of copepods was studied in graduated cylinders placed near an open window, with additional illumination from a window and open door on the other side of the room. The light intensity was measured at the wall of the cylinder both toward and away from the nearer window.

Cylinders of 1000-ml. capacity were used for *Calanopia* and *Acartia. Paracalanus,* which is smaller, was studied in 100-ml. cylinders. The distribution of specimens in the water column was determined over a 24-hour period from counts made at intervals at ten levels, from the surface to the bottom. The graduations on the cylinders facilitated these counts, but the distribution was expressed in mm. depth. During the night, counts were made quickly using a flashlight. Such brief illumination did not seriously affect the migration.

In studying the influence of xanthene dyes, the vertical distribution was followed similarly in cylinders containing rhodamine B or fluorescein sodium in sea water.

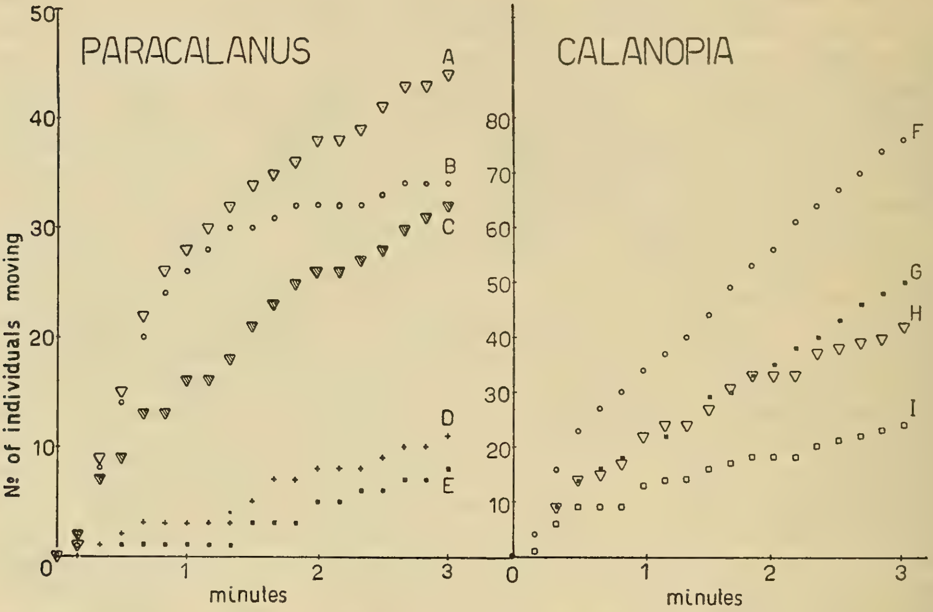

FIGURE 1. Phototaxis of *Paracalanus crassirostris* and of *Calanopia americana* to a point source of light. One experiment is shown for each species. Other experiments gave similar results. *Curves A and F*—Total numbers of animals moving in sea water without respect to direction of movement. Number of individuals moving was determined every 10 seconds. The curves indicate cumulative totals through each 10-second interval. *Curves B and G*—Photopositive reactions in sea water. *Curves C and H*—Total numbers of animals moving in rhodamine B (8.4 × 10^{-6} *M*) without respect to direction of movement. *Curve D*—Numbers of animals exhibiting somersaulting in rhodamine B (8.4 × 10^{-6} *M*). *Curves E and I*—Photopositive reactions in rhodamine B (8.4 × 10^{-6} *M*). Temperature 26.0° C.

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RESULTS

Phototactic responses of Paracalanus

Specimens of *Paracalanus* showed both photopositive and photonegative responses to a point source of light. A measure of these tactisms was made by counting individuals of a randomly distributed population moving toward or away from a light source after sudden exposure to light. In Figure 1 it will be seen that the number of positive phototactisms is large at first, as shown by the coincidence of the initial slopes of curves for total animals moving (curve A) and for animals exhibiting a photopositive reaction (curve B). After about one minute, the number of photopositive reactions per unit time decreased as animals collected toward the light, and the number of photonegative reactions increased as some of the animals reversed their direction and moved away from the light. Clearly, the percentage of animals exhibiting a photopositive reaction is a function of time and the experimental arrangement. If the photopositive animals had been removed after the initial response, essentially no photonegative responses would have been observed.

The percentage of animals showing photopositive reactions during a three-minute trial was less at night than during the day but the difference was not statistically significant (Table I).

TABLE I

Response of copepods to a point source of light

	Acartia lilljeborgi		*Calanopia americana*		*Paracalanus crassirostris*	
Night	56.5 ± 2.1	<0.01	67.1 ± 10.9	>0.50	58.0 ± 26.7	>0.05
Day	81.5 ± 6.0		60.6 ± 6.8		79.0 ± 2.2	

The figures show the mean percentage and standard deviation of animals exhibiting photopositive reactions as compared with other reactions (photonegative reactions and somersaulting). Values using Student's test for probabilities that night and day responses are alike are also given.

Effects of rhodamine B and fluorescein on Paracalanus

Rhodamine B (8.4×10^{-6} M) produced three effects on the behavior of *Paracalanus:*

(1) The number of animals moving was significantly decreased (Table II, column A, Fig. 1, *cf.* curves A and C). The inhibition was readily reversed by returning the animals to sea water. There was no statistically significant difference in movement before and after exposure to rhodamine B.

(2) A somersaulting reaction was induced. The extent of this behavior is illustrated in Figure 1 (*cf.* curves A and D). Movements returned to normal in sea water.

(3) The *relative* number of photopositive reactions was decreased (Table II, columns B and C, Fig. 2). The effect was readily reversed on removal of rhodamine B (Fig. 2).

Fluorescein sodium (1.1×10^{-5} M), in contrast to rhodamine B, did not significantly alter normal activity or phototactic reactions (Fig. 2).

Wave-length as a possible factor in rhodamine B effects

Baylor and Smith (1957) and Dingle (1962) observed that colored lights affected the movements of copepods. Since rhodamine B is bright red in solution, there was need to determine whether the effects of the dye were due to the screening out of certain wave-lengths or to its chemical action. Action due to screening was tested by placing a solution of rhodamine B of the same concentration and path length as the usual experimental solution between the light source and a chamber of sea water containing the specimens. No effect was noted. If wave-length only is concerned in the action of rhodamine B, immediate effects would be expected on placing the animals in it. This was tested by counting the total numbers of animals moving across the field of the microscope beginning one minute after the addition of rhodamine B and during a second period about 10 minutes later. The rhodamine B effect was not maximal during the first minutes, indicating a factor other than the color of the solution.

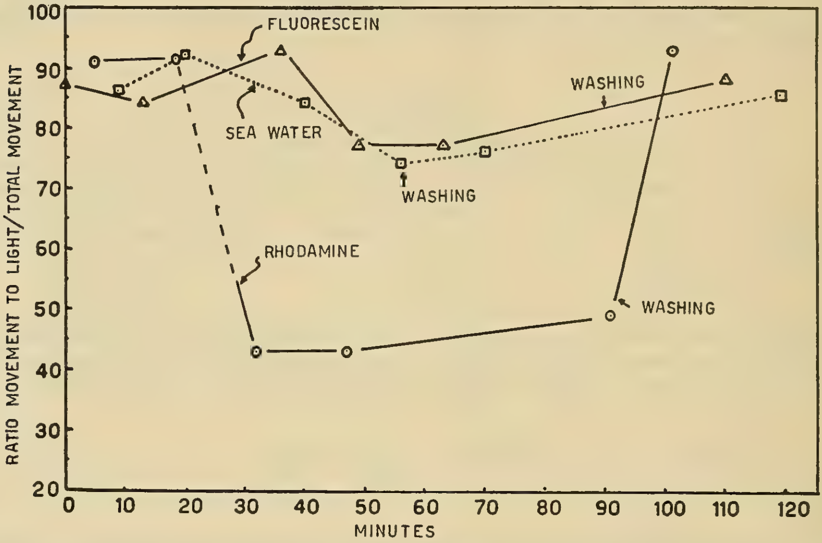

FIGURE 2. Effects of rhodamine B and fluorescein sodium on phototaxis of *Paracalanus crassirostris*. One experiment is shown. Other experiments gave similar results. Rhodamine B (8.4×10^{-6} *M*) decreased the relative number of photopositive tactisms and induced somersaulting. After removing the rhodamine B the ratios became normal again. Fluorescein sodium (1.1×10^{-5} *M*) was without significant effect. Total numbers of specimens: control, 55; rhodamine B, 64; fluorescein sodium, 53. Temperature, 22.1° C.

Effects of pyronine B on Paracalanus

Pyronine B (8.4×10^{-6} *M*) diminished the number of *Paracalanus* moving. In three experiments the percentages of moving animals in pyronine B after 10 to 20 minutes treatment were 21, 35, and 47, as compared with the numbers moving prior to addition of the compound. Many of the animals lay motionless in pyronine B. The magnitude of the inhibitory effects of pyronine B and rhodamine B on movement was similar. The recovery of movement in sea water was less rapid and less complete after treatment with pyronine B (*cf.* Figs. 2 and 3). Some specimens in pyronine B showed a lateral jumping or zigzag jerking movements not seen in rhodamine B. In two experiments this movement was still evident on the day following removal of the pyronine B. In 1.7×10^{-6} *M* pyronine B the same abnormal movements occurred without a depression of general activity (one experiment).

Phototactic responses of Calanopia

Calanopia showed phototactism but differed from *Paracalanus* in its responses. On exposure to a point source of light, individuals swam to and fro continuously, giving a nearly constant number of photopositive and photonegative reactions per unit time. This behavior is expressed as a nearly constant slope in curves of animals showing positive phototaxis (Fig. 1, curve G) and for total animals moving (Fig. 1, curve F) after the initial 30 seconds. The general activity of *Calanopia* was reduced in the daytime but the percentage of animals giving photopositive reactions was not significantly different statistically during day and night (Table I).

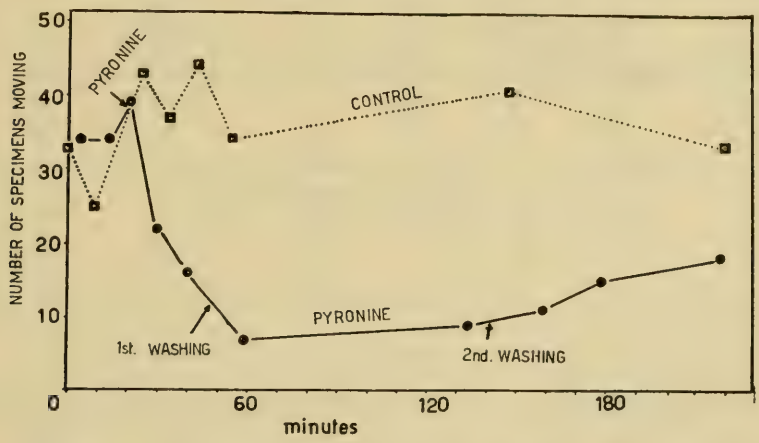

FIGURE 3. Effect of pyronine B on locomotion of *Paracalanus crassirostris*. Note absence of rapid and complete reversibility on washing. *Cf.* Figure 2. Pyronine B (8.4×10^{-6} *M*). Total numbers of specimens: control, 80; pyronine B, 80. Temperature, 21.5°–22.5° C.

Rhodamine B (8.4×10^{-6} *M*) brought about a reversible decrease in the number of animals moving (Fig. 1, curves F and H; Table II, column A) but increased the relative numbers giving photopositive reactions (Table II, columns B and C). There were no somersaulting movements such as those seen in *Paracalanus*. Fluorescein sodium (1.1×10^{-5} *M*) was without effect.

Phototactic responses of Acartia

Acartia, on exposure to a point source of light *at night*, responded in the manner of *Calanopia* but differed from both *Calanopia* and *Paracalanus* in showing a significantly higher percentage of photopositive reactions *during the day* (Table I).

In rhodamine B (8.4×10^{-6} *M*) a reversible decrease in movement occurred, but there was a relative increase in the number of photopositive reactions as in *Calanopia* (Table II, columns B and C). Rhodamine B abolished the difference

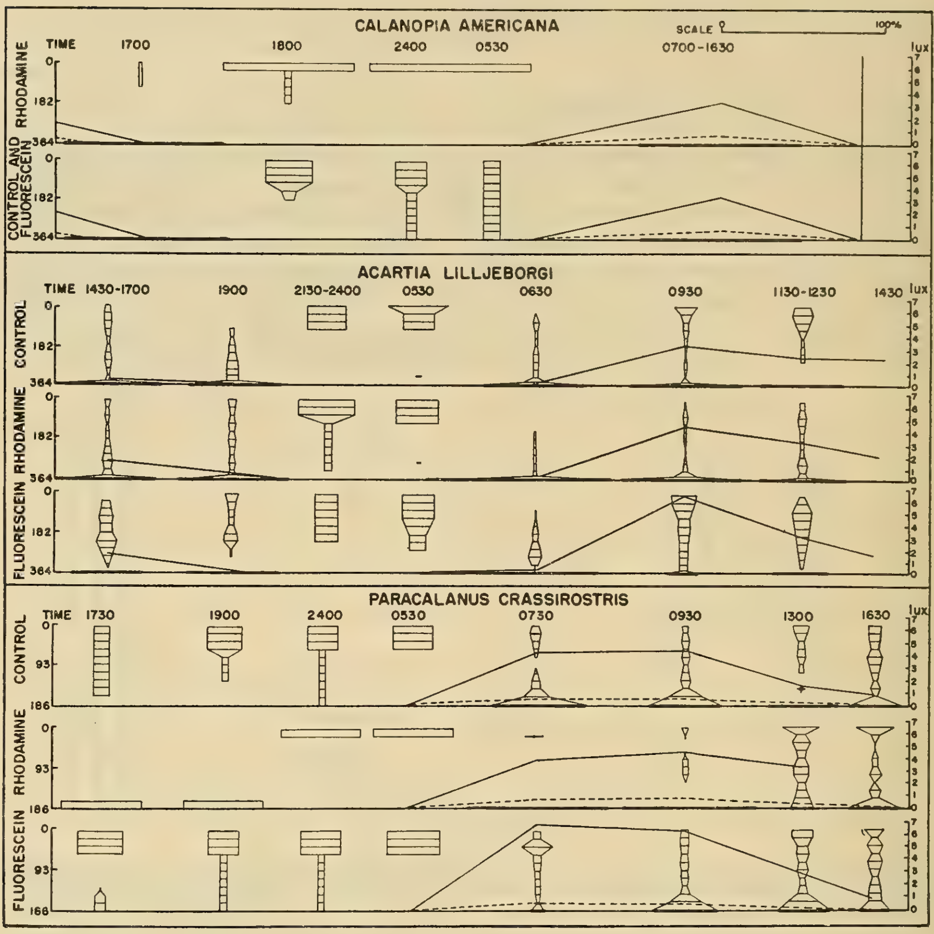

FIGURE 4. Vertical migration of *Paracalanus crassirostris, Acartia lilljeborgi* and *Calanopia americana.* The numbers of animals at various levels in glass cylinders were determined at intervals through the day and night. The numbers across the top of each diagram show the time. Each small diagram shows the percentage distribution at a particular time at different levels of the water column (see percentage scale at top right). Water levels are indicated in mm. on the left. Light intensity is indicated in lux on the right. The curves show the light intensity during daylight hours; the solid line gives the intensity on one side of the cylinders and the broken line on the other. For *Acartia* the illumination at 1430 is indicated on the right and the illumination at 1700 at the left side. The heavy line at zero level represents the percentage of animals at the bottom. Total number of individuals: *Paracalanus,* control—113; rhodamine B—70; fluorescein sodium—104; *Acartia,* control—235; rhodamine B—434; fluorescein sodium—147; *Calanopia,* control—195; rhodamine B—240; fluorescein sodium—152. The number of animals used varied considerably, as shown. Experiments in which different numbers were used (usually smaller than these) gave similar results. Temperatures: *Paracalanus,* 18.0°–24.0° C; *Acartia,* 12.0°–23.5° C; *Calanopia,* 18.0°–25.0° C. It is to be noted that the temperature range was considerable during some diurnal cycles and series of experiments. However, individual experiments carried out over smaller, and different, ranges (18.0°–23.0° C; 22.1°–27.5° C.) gave similar results.

in photopositive reactions during the day and at night. Fluorescein sodium (1.1×10^{-5} M) was without effect; and in an isolated experiment the same was true at 1.1×10^{-4} M, which was 13 times the concentration of the rhodamine B solution.

Migratory behavior

Diurnal migratory behavior of *Calanopia, Acartia,* and *Paracalanus* was observed in vertical cylinders in diffuse light. Figure 4 illustrates the findings. The length of the horizontal lines at the times observed is proportional to the percentage of animals at each depth (ordinate).

TABLE II

Effects of rhodamine B on locomotion and phototaxis of copepods

Species	A Mean % of total reactions in rhodamine B	B Mean % of positive phototaxis in rhodamine B	C Mean % of positive phototaxis in sea-water control	D Probability of similarity between B and C
Paracalanus crassirostris	64	53	79	<0.01
	<0.01	<0.01	<0.01	
Calanopia americana	51	83	67	<0.05
	<0.01	>0.70	1.00	
Acartia lilljeborgi	46	81	68	<0.01
	<0.01	<0.05	>0.20	
Paracalanus crassirostris	64	53	79	<0.01

Probabilities of similarities between species for responses given in headings of columns A, B, and C were calculated by Student's test. *Paracalanus* appears twice for the purpose of statistical comparisons between species. *Column A* was calculated from the number of total reactions (photopositive, photonegative, and somersaulting) in rhodamine B (8.4×10^{-6} M) as compared with total reactions in sea water immediately prior to the addition of rhodamine B. The usual period of treatment in rhodamine B was 30 minutes or less. *Column B* was calculated from the number of photopositive reactions in rhodamine B as compared to total reactions (photopositive, photonegative, and somersaulting). *Column C* was calculated from the number of photopositive reactions in the sea-water control as compared with the total reactions (photopositive, photonegative, and somersaulting).

Calanopia

The diurnal migration cycle of *Calanopia* was sharply defined. In the late afternoon, night, and early morning the animals accumulated in the upper layers or were evenly distributed vertically throughout the water column. They then migrated to the bottom and remained on the side of lower light intensity until the upward migration in the late afternoon. The upward migration occurred somewhat earlier in rhodamine B (8.4×10^{-6} M) and the animals accumulated at the top of the water column to a greater degree than in sea water alone. Fluorescein sodium (1.1×10^{-5} M) was without significant effect.

The earlier upward migration in the red rhodamine B solution could be due to a decrease in light intensity at certain wave-lengths. A simple experiment was carried out to test this. The experimental animals were placed in a glass cylinder (100 ml.) containing sea water inside a larger cylinder (1000 ml.) containing rhodamine B solution (8.4×10^{-6} M) or sea water. The control animals were placed in similar cylinders, both containing sea water. In this way, animals in sea water were exposed to natural light filtered through rhodamine B solution or filtered through sea water. The experimental animals exposed to light filtered through rhodamine B solution accumulated at the bottom in the afternoon whereas the control animals were found randomly distributed in the water column. This experiment demonstrated that the earlier upward migration in rhodamine B solution is not due to the effect of certain wave-lengths. It also showed that light passing through a solution of rhodamine B caused a downward migration of animals at a time of day when they were normally distributed through the water column.

Acartia

The diurnal migratory cycle of *Acartia* was different in adults and young forms (copepodites). At night the adults were in the upper third of the water column (Fig. 4) and then migrated to the bottom in the morning and remained on the side of higher light intensity during the day. The young forms were dispersed during the day (Fig. 4). In the early morning they were distributed through the lower two-thirds of the column. This was followed by a migration to the upper layers later in the morning and a return downward in the afternoon. They moved to the upper portion of the water column with the adults at night.

Rhodamine B (8.4×10^{-6} M) increased the vertical dispersion of young forms at midday, late afternoon, and night. However, during the morning, rhodamine B brought about a higher accumulation of both adults and young at the bottom (Fig. 4). In fluorescein sodium (1.1×10^{-5} M) the upward migration of late afternoon occurred sooner than in sea water alone.

Paracalanus

Paracalanus exhibited a less marked diurnal migratory cycle than *Calanopia* and *Acartia*. Specimens were dispersed vertically through the water column at most hours (Fig. 4). The major accumulation at night and in the very early morning was in the upper layer. Then, later in the morning and also under intense natural illumination, most of the animals migrated to the bottom. (When light intensities exceeded 3229 lux the animals were found on the darker side of the cylinders.) In the afternoon the animals moved upward.

Rhodamine B (8.4×10^{-6} M) exerted a marked effect in bringing about an accumulation of animals at the surface at night and at the bottom in the morning and late afternoon, but not in the early afternoon. The effect of rhodamine B was to reduce vertical dispersion. The behavior of *Paracalanus* resembled that of *Calanopia* in rhodamine B; in sea water the behavior of these species was distinctly different.

Behavior in continuous darkness

Harris (1963) reported that *Daphnia magna* and *Calanus helgolandicus* continued their diurnal migration cycle in darkness. In order to determine the effect of the absence of light on migratory behavior, specimens of *Calanopia* and *Acartia* were placed in a cylinder in dark room and observed concurrently with specimens exposed to natural illumination. Observations of animals in the dark were quickly made with a flashlight. A brief exposure to light has been shown to be without significant effect on the migratory cycle (Harris, 1963; Harris and Wolfe, 1955). *Paracalanus* was not tested because it did not have a marked migration.

Animals in the dark did not migrate. This is in agreement with the behavior of *Acartia tonsa* (Conover, 1956). Throughout a period of 24 hours or longer, *Calanopia* was evenly dispersed vertically in the water column, whereas *Acartia* remained near the surface. These distributions are characteristic of these species at night. When a cylinder containing *Acartia* was transferred from the dark to diffuse natural illumination in midmorning, the animals immediately migrated to the bottom where these copepods are normally found in diffuse daylight at this time of day. A rapid downward migration could also be brought about at night by exposing the animals to a light above the water column.

A direct comparison between the migration in the natural environment and movements of the animals in vertical cylinders in the laboratory was carried out in the following way. During the period January 30th to February 22nd, samples were taken every 4 to 6 hours from the surface water at the pier at Ubatuba. At almost the same time readings were made of the distribution of individual species in the cylinders. *Calanopia* and *Acartia* were caught in the surface samples only at night and before sunrise. *Paracalanus* occurred in surface samples both at night and during the day. These results correspond to the findings on migration in the laboratory (Fig. 4).

DISCUSSION

The responses of *Acartia, Calanopia,* and *Paracalanus* to a point source of light differ. The differences are of degree. *Calanopia* on exposure swam to and fro, toward and away from the light. *Acartia* behaved similarly but swam less rapidly. In *Paracalanus* the alternating movements were much reduced; positive phototaxis was accordingly more strongly expressed.

This behavior is probably related to the mode of feeding. *Calanopia,* the most active, is also the most predaceous, and cases of cannibalism among adults were observed during rearing experiments (Björnberg, personal communication). *Acartia,* intermediate in its phototactic movements, lives on larger particles (see Mullin, 1966) and larvae caught by grasping or raking movements (Conover, 1956). *Paracalanus,* the slowest in locomotion, is a fine-particle grazer and feeds merely by creating a current through continuous vibration of the head appendages.

The diurnal migratory cycle observed in vertical cylinders differed in the three species. The differences could be advantageous to feeding. The downward migration of *Calanopia* during daylight, also observed by Clarke (1934) and Johnson (1938), brings it to the bottom in shallow water where the zooplankton is known to accumulate during the day. The finer particles in suspension are found at all depths in shallow water; and, interestingly, *Paracalanus* and the younger *Acartia,* which are fine-particle grazers, show less well defined migration to the bottom than adults. Conover (1956) observed diurnal distribution in *Acartia tonsa* and *Acartia clausi* in colder waters. He found accumulation of *A. tonsa* in the surface layers at night and accumulation at the bottom in the morning. A difference in distribution for adults and young of this species was also noted.

A quantitative measure of species differences is given by their phototactic responses (Table II, C). *Paracalanus* and *Calanopia* were significantly different, but differences between *Calanopia* and *Acartia* and between *Acartia* and *Paracalanus* were not significant. However, in rhodamine B (Table II, B) *Acartia* and *Paracalanus* did respond differently.

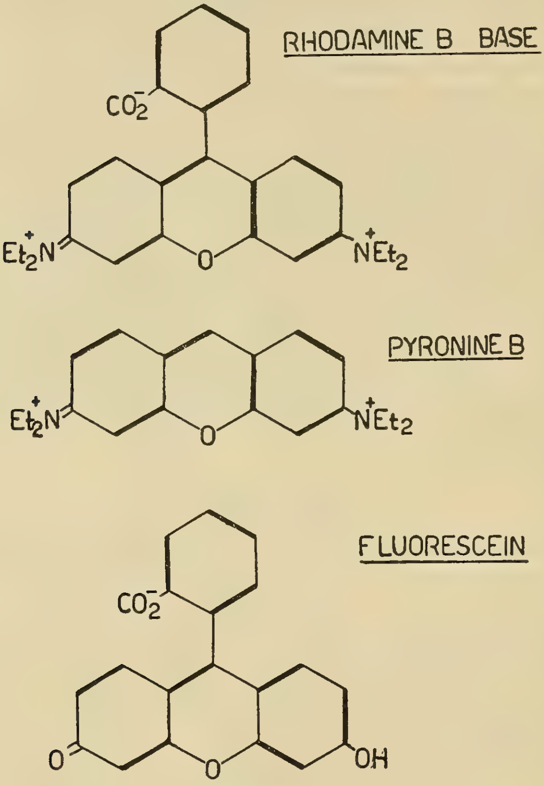

FIGURE 5.

Rhodamine B had the general action of inhibiting locomotion in all three species of copepods in response to a point source of light. Under natural illumination rhodamine B restricted dispersion in a vertical water column (except in young *Acartia*) without altering general migration. The more restricted vertical dispersion probably resulted from a reduction of locomotor movements. In the case of *Paracalanus,* the effect of rhodamine B is to change the distribution in the water column by bringing about a concentration of animals in the surface layers or at the bottom except during the period 0730–1630 (Fig. 4). Rhodamine B has thus induced a behavior pattern in *Paracalanus* which resembles *Calanopia* for the major part of the daily cycle.

The selectivity of the action of rhodamine B was striking. It induced abnormal locomotion in *Paracalanus* but not *Acartia* or *Calanopia.* It increased the percentages of photopositive responses in *Acartia* and *Calanopia* but decreased the percentage in *Paracalanus.* It increased vertical dispersion of young *Acartia* but not of adults. These results suggest that interesting approaches to differences between species and between developmental stages of marine invertebrates might be made through the use of pharmacological agents.

The physiologically effective xanthenes, rhodamine B and pyronine B, have two diethylamine groups (Fig. 5). Fluorescein, in which these groups are replaced by oxygen and a hydroxyl group, was essentially inactive on copepods. Pyronine B, which differs from rhodamine B in lacking the ring bearing the carboxyl group, caused abnormal jerking movements not evident with rhodamine B.

Studies of toxicity and de-ethylation of rhodamine (Webb and Hansen, 1961; Webb *et al.,* 1961) and the effects of pyronine, including a slight photodynamic action (Pearse, 1953; Nagai, 1962; Herzberg *et al.,* 1963; Wacker *et al.,* 1963), are not very helpful in interpreting effects of these compounds on copepods. Possible loci of action of rhodamine B include photoreceptor cells, central nervous system, neuromuscular junction, and muscle. The somersaulting in *Paracalanus* in rhodamine B suggests central nervous system involvement. The movements apparently resulted from an increased tonus of at least one set of muscles which brings about a maintained downward flexing of the abdomen and a hook-like form. This action would be consistent with a relative increase in firing of certain central pathways or with the blocking of impulses to certain groups of muscles, with the result that the antagonistic muscles are maintained in contraction. Blocking seems more likely than preferential stimulation of pathways since general locomotor movements are reduced by rhodamine B. It is of interest that somersaulting, induced in *Paracalanus* by rhodamine B, is the normal form of progression observed in the nauplii of this and other calanoid species with hook-form larvae (Björnberg, unpublished).

The action of rhodamine B on movements of *Calanopia, Paracalanus,* and *Acartia* was quickly reversible. The slower and incomplete reversibility of inhibition of movement by pyronine B could be related to a difference between the compounds in binding by cytoplasmic constituents.

SUMMARY

1. Phototaxis of the copepods *Paracalanus crassirostris, Calanopia americana,* and *Acartia lilljeborgi* has been measured by determining the percentage of a population moving toward or away from a point source of light per unit time. Quantitative differences in positive phototaxis were found between the species. Photopositive responses differed during the day and night in *Acartia* but not in *Paracalanus* and *Calanopia.*

2. Rhodamine B (8.4×10^{-6} *M*) brought about the following effects: (a) Locomotor activity was reversibly inhibited in all species. (b) Photopositive responses were increased in *Calanopia* and *Acartia* but decreased in *Paracalanus.* (c) The difference between day and night responses to a point source of light was abolished in *Acartia* and induced in *Calanopia.* (d) Somersaulting was induced in *Paracalanus* but not in the other species.

3. Pyronine B (8.4×10^{-6} *M*) also decreased locomotor activity. Fluorescein sodium (1.1×10^{-5} *M* and 1.1×10^{-4} *M*) was without significant effect.

4. *Paracalanus, Calanopia,* and *Acartia* exhibited characteristically distinct diurnal migratory cycles in vertical cylinders, which correlated well with behavior in natural waters. *Calanopia* and *Acartia* migrated to the bottom in the daylight whereas *Paracalanus* and young forms of *Acartia* were widely distributed vertically during daylight. Specimens of *Calanopia* and *Acartia* kept in the dark did not migrate.

5. The effects of rhodamine B (8.4×10^{-6} *M*) on vertical migration depended upon species, developmental stage, and time of day. In general, rhodamine increased the concentration of animals at the surface at night and at the bottom in daylight. Fluorescein sodium (1.1×10^{-5} *M* and 1.1×10^{-4} *M*) had little effect on vertical migration.

6. The effectiveness of rhodamine B and pyronine B is probably related to the presence of diethylamine groups lacking in fluorescein.

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PHOTOSYNTHETIC CAPACITIES AND PHOTOSYNTHETIC ACTION SPECTRA OF ENDOZOIC ALGAE OF THE MASSIVE CORAL FAVIA

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Many corals and clams in the Indo-Pacific area are inhabited by brown symbiotic algae. In some corals these intracellular symbiotic algae give the impression of a dense surface-layer chocolate smear. These algae are called zooxanthellae. They are dinoflagellates and resemble *Symbiodinium microadriaticum* Freudenthal. When on corals and clams, and also immediately after separation from the host, the dinoflagellates are non-motile. They possess the typical dinoflagellate furrows (for review see McLaughlin and Zahl, 1966). When left stirred in filtered sea water for about one day, they start swimming in a way typical for a dinoflagellate (Haxo, unpublished data). The pigment composition from zooxanthellae is also characteristic of the dinoflagellate group (Jeffrey and Haxo, unpublished data) and the *in vivo* absorption spectrum has the typical shape of absorption spectra from dinoflagellates (Shibata, unpublished data; Halldal, 1958a). In the massive coral *Favia* this symbiotic dinoflagellate constitutes a dense cover that captures nearly all the light that hits the coral. The average light conditions at different wave-lengths within the coral have been measured (Shibata and Haxo, unpublished data), and results from such analyses are schematically illustrated in Figure 1. At most 0.1% of the light between 340 and 680 nm penetrates the living part of the coral which has the zooxanthellae. Around 700 nm about 1% at the most may be left, and at and above 720 nm 2% or more. Shibata and Haxo also showed that the light intensity rapidly decreased with depth within the dead part of the coral. Only a fraction of the light hitting the coral was left at around 1 cm. below the surface.

Under these light conditions underlying green layer algae can live and photosynthesize. These algae seem to be of mixed genera and species, most of them probably belonging to the genus *Ostreobium* within the order Siphonales. In *Favia* they were present as a green curved layer 0.5–1 cm. thick; the layer penetrated from the living part of the coral to a depth of 1–3 cm. into the dead part (see Figs. 1 and 6). These algae were present in around 5% of the *Favia* specimens collected. At times several less pronounced layers occurred below this upper layer. Pigment analyses from these deeper areas revealed chlorophyll degradation products (Jeffrey, unpublished data), and photosynthetic activity could as a rule not be demonstrated. These facts indicate that green layers or patches below the green layer nearest the surface of the coral constitute or include dead algae. Pigment analyses of the green layer nearest the surface showed a composition typical of a Siphonales (Jeffrey, unpublished data). Jeffrey also noted that the alga was very rich in chlorophyll *b*, which constituted $\frac{2}{3}$ of the chlorophyll *a* content.

This paper presents light-intensity/response analyses, and action spectra determinations of the symbiotic dinoflagellate and the endozoic Siphonales.

MATERIALS AND METHODS

The coral *Favia pallida* Dana. This coral was the source of algal material for experiments presented in this paper. In several cases checks were made on samples taken from similarly shaped corals. As examples were selected *Astreopora ocellata* Bernard, *Goniastrea mantonae* Crossland, *Platygyra rustica* Dana, *Porites lutea* Milne Edwards and Haime. In no cases could be demonstrated deviations from photosynthetic measurements obtained with algae collected from *Favia.*

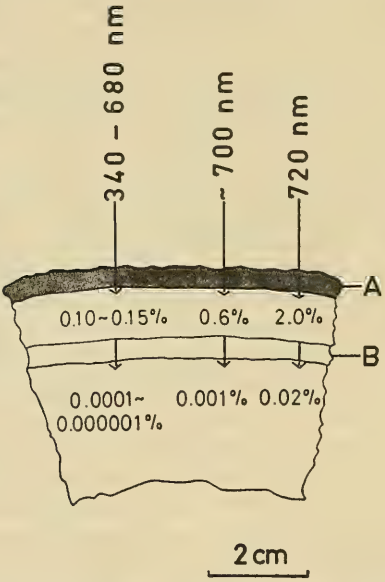

FIG. 1. Schematic drawing of light conditions within the massive coral *Favia* according to measurements of Shibata and Haxo (unpublished data). A. The living part of the coral with the zooxanthellae. B. The *Ostreobium* layer.

The dinoflagellate cf. *Symbiodinium microadriaticum* (Zooxanthellae). This alga can be isolated from corals and clams in different ways. Muscatine (1967) developed a method related to the one used in preparing chloroplasts and chloroplast fragments for Hill reaction studies. Provasoli (personal communication) successfully isolated the algae with a brush. Both these methods, or adaptations of them, were employed in the present investigation. However, both for action spectra analyses and for intensity/response determinations only a tiny sample was needed, enough to cover the about 0.6 mm.^{2} surface of the oxygen sensor cathode used. As a rule samples were therefore directly removed from the coral by a needle or a pair of forceps, and immediately placed upon the oxygen sensor cathode (see below and Fig. 2).

The Siphonales *Ostreobium.* For the studies of the green layer alga of *Favia* a coral specimen was cut in dim light. A small sample of the green-colored lime from the inner part of the coral was removed with a sharp knife or a razor blade. This piece was then further cut to a small thin fragment about 1 × 1 mm.[2], and directly fastened to the oxygen sensor cathode (see below and Fig. 2).

Oxygen measurements. These were performed by means of a Beckman oxygen sensor adapted to a Beckman model 76 pH meter, and the signal recorded with a Texas Instrument Incorporated Servoriter Recorder. The algal sample was kept in place on the gold cathode by means of a black tape ring whose central hole was covered with a piece of dialyzing diaphragm to allow for access of light and oxygen exchange between the sample and the surrounding water reservoir (Fig. 2). The volume between the diaphragm and the oxygen sensor cathode was filled with filtered sea water. During experiments the oxygen sensor with the sample was inserted in a cup with 1.5 ml. filtered sea water as outer reservoir. The cup with the sea water was rotated at 72 revolutions per minute. The bottom of the cup was a quartz window to allow for transmission of irradiation in the visible and ultraviolet region of the spectrum (see Halldal, 1964).

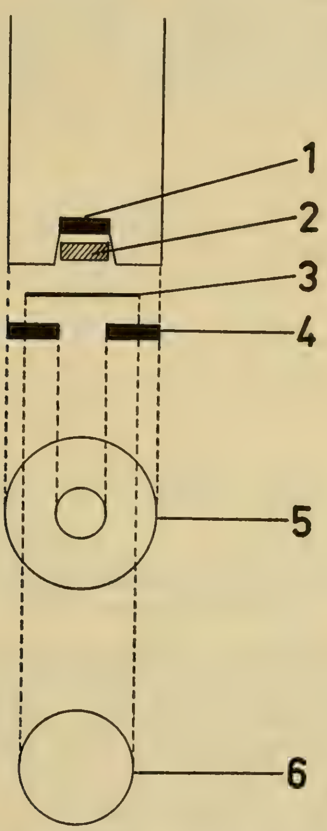

FIGURE 2. The mounting of the algal sample (2) onto the gold cathode (1) of the Beckman oxygen sensor was achieved through the dialyzing membrane (3) and (6), which was fastened to the oxygen sensor with a black tape ring (4) and (5).

Irradiations. These were performed with an automatic recording field instrument for measuring action spectra of photosynthesis (Halldal and Nestor, unpublished data). The principle of such automatic recording of photosynthetic action spectra is described by French, Myers and McLeod (1960). With such apparatus equal numbers of quanta can be delivered at different wave-lengths during a spectrum sweep, and the photosynthetic pO_2 directly recorded against wave-lengths. The apparatus has also been constructed to record the action spectrum during a sweep where the pO_2 is kept constant at different wave-lengths. This last mentioned method was not used in the present investigation. This apparatus is limited to wave-lengths between 400 and 720 nm. In the far red, and also in the near and far ultraviolet region of the spectrum, irradiations were performed with a Bausch and Lomb 250 mm monochromator. The action spectra determinations were partly done by automatic sweeps, partly by point measurements using methods described in earlier papers (Halldal, 1964, 1966). Radiant energy was measured with an Eppley thermopile in combination with a Keithley nano-volt meter. A spectral half-band width of 10 nm was used during the measurements.

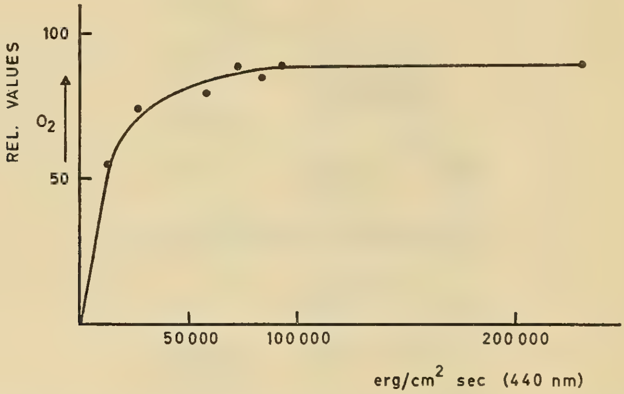

FIGURE 3. Intensity/response curve for the symbiotic dinoflagellate (Zooxanthellae) isolated from the massive coral *Favia.*

RESULTS

The dinoflagellate (Zooxanthellae)

Light intensity/response curve. The light intensity/response curve for this alga is given in Figure 3. At 440 nm the response was linear with intensity to 1500 erg. cm.$^{-2}$ sec^{-1} and saturation was obtained at 95,000. The intensity of monochromatic irradiation that caused photosynthetic inhibition could not be obtained with the equipment available (225,000 erg. cm.$^{-2}$ sec^{-1}).

Action spectrum. The action spectrum of photosynthesis is presented in Figure 4. This alga responded with photosynthetic oxygen evolution to radiation between 300 and about 720 nm. The action spectrum reveals the chlorophyll *a* peaks at 440 and 675 nm. An inflexion at 640 nm indicates the participation of chlorophyll *c* in photosynthesis. The shoulder at 490 nm reflects the peridinin and the chlorophyll c peak combined in this spectral region. At about 540 nm a small but distinct maximum (sometimes inflexion) was always noticeable. This detail in the action spectrum of photosynthesis corresponds to a similar band at 540 nm in the *in vivo* absorption spectrum of zooxanthellae (Shibata, unpublished data). During analyses of the excitation spectrum of fluorescence of *in vivo* samples of zooxanthellae a distinct peak corresponding to this wave-length was observed (Shibata, unpublished data).

As the magnitude of this peak (inflexion) showed variations, presumably caused by different degree of excitation, it seems as if this dinoflagellate undergoes changes in its absorption around 540 nm. Further analyses of this problem were not performed during the investigation on board the Alpha Helix in the Great Barrier Reef area in 1966.

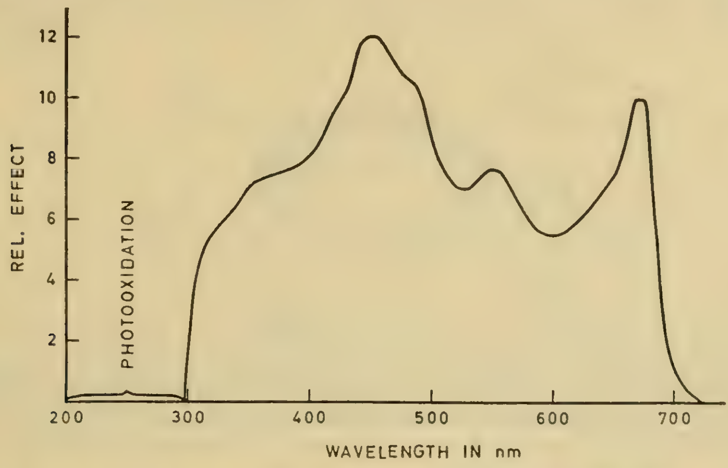

FIGURE 4. Action spectrum of photosynthesis of the symbiotic dinoflagellate isolated from the massive coral *Favia.*

The Siphonales Ostreobium

Light intensity/response curves. This alga reached saturation, and also photooxidation, at very low light intensities. It was also demonstrated that the position of the alga within the coral was of utmost importance for its response to light. This is demonstrated in the series of curves presented in Figure 6. In Figure 5 are marked the sampling positions of three fragments containing *Ostreobium.* The intensity/response curves are given in Figure 6, where the numbered curves correspond to the marked positions of Figure 5. It was shown that a sample taken from the top of the green layer (/1/ in Fig. 6) showed linear response with intensity to 1000 erg. cm.$^{-2}$ sec^{-1} at 440 nm, and no photosynthetic inhibition was noted at 5000 erg. cm.$^{-2}$ sec^{-1}. A sample taken from within the middle part of the green layer (/2/ in Fig. 6) showed linear response with intensity to about 100 erg. cm.$^{-2}$ sec^{-1}, saturation between 100 and 700 erg. cm.$^{-2}$ sec^{-1}, reduced photosynthetic activity between 700 and 1800 erg. cm.$^{-2}$ sec^{-1}, and photooxidation above this intensity. Finally, a sample taken from the bottom of the green layer (/3/ in Fig. 6) showed photooxidation at all intensities applied. Samples taken from layers or patches of *Ostreobium* below the layer nearest the surface did usually not respond to light. In some few cases photooxidation was observed.

Action spectra. Action spectra of both photosynthesis and photooxidation could be performed. In the case of photooxidation the reduction in pO_2 was reversible with gentle doses of irradiation. Because of this, action spectra of photooxidation could be recorded with the field apparatus, though corrections and adjustments were needed (see Fig. 8).

The action spectrum of photosynthesis is presented in Figure 7. It was shown that *Ostreobium* in the ultraviolet responded with photosynthesis down to 300 nm, that is, the same spectral region in the ultraviolet which was photosynthetically effective for zooxanthellae. Below 300 nm photooxidation occurred during exposures of more than 15 seconds duration of moderate intensity (about 500 erg. $cm.^{-2}$ sec^{-1}, but highly dependent upon the sample; see above). However, the far ultraviolet response of this alga gave a rather complicated picture, evidently highly dependent upon sample and radiation intensity. Both very rapid increases and decreases in pO_2 were observed. These fast changes were always succeeded by a slower degree of photooxidation. Data are available from several such far-ultraviolet experiments, but it is uncertain whether they suffice for analysis of the rather complicated response.

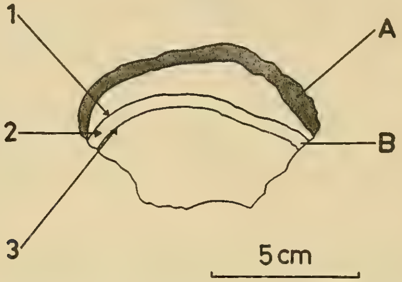

FIGURE 5. Schematic drawing of a cut from the massive coral *Favia*. A. Living part of coral with zooxanthellae. B. Green layer of the Siphonales *Ostreobium* within the dead part of the coral. The numbers illustrate positions of sampling, which correspond to the numbered curves in Figure 6.

Above 300 nm photosynthesis occurs. Small, but significant, inflexions are noticeable around 310 and 340 nm. At 440 nm the chlorophyll *a* peak in the blue is apparent, and participation of chlorophyll *b* and carotenoid(s) is reflected by the shoulder at 480 nm. Going towards longer wave-lengths the shape of the curve reflects quite well what is to be expected from an action spectrum plot of photosynthesis in a green alga. The relatively high response at 645 nm should, however, be pointed out. This reflects the relatively high chlorophyll *b*/chlorophyll *a* ratio in the pigment composition of this alga (Jeffrey, unpublished data).

Above 680 nm some important deviations from what is usually found in green, and also in other algae, are clearly noticeable. Usually action spectra of photosynthesis in green algae show a rapid falloff toward 700 nm, and the response at 720 nm is as a rule negligible. The endozoic *Ostreobium* taken from within the

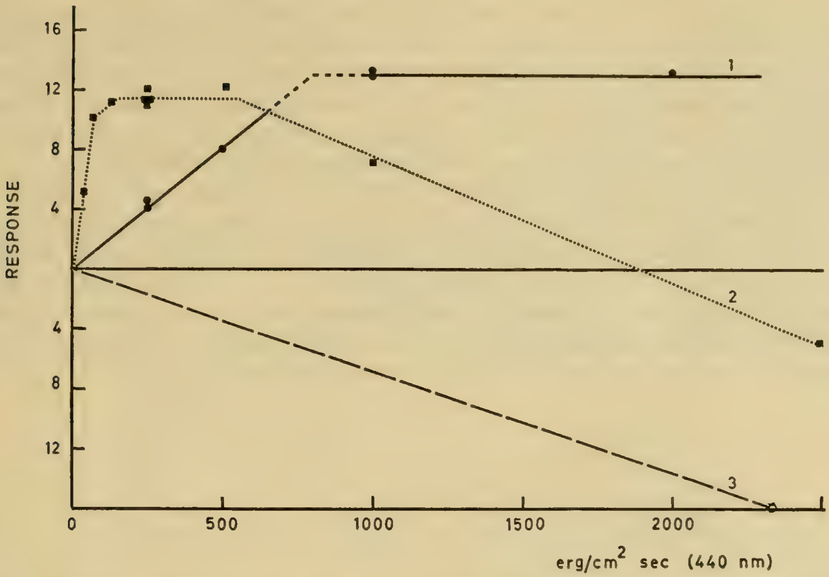

FIGURE 6. Intensity/response curves for the endozoic Siphonales *Ostreobium* taken from within the massive coral *Favia*. For sampling position see Figure 5.

massive coral *Favia,* where it has been exposed to the highly distorted light field outlined earlier (see Fig. 1), showed a response to 700 nm radiation that was practically the same as that at 680 nm, and the response at 720 nm was usually between 70 and 80% of the maximum at 680 nm. The action spectrum curve dropped rapidly from 720 nm toward longer wave-lengths, but even at 750 nm the oxygen evolution was easily measurable.

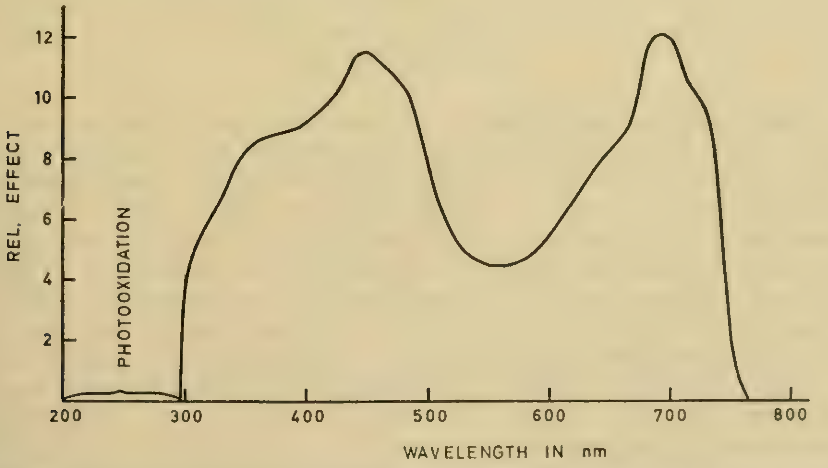

FIGURE 7. Action spectrum of photosynthesis for the Siphonales *Ostreobium* isolated from within the coral *Favia*.

The decrease from 700 nm toward longer wave-lengths is, as shown in Figure 7, interrupted by a distinct shoulder. This detail strongly indicates the presence of a pigment with an absorption band at this wave-length. The magnitude of this shoulder in the action spectrum occasionally showed some variations. It was assumed that these variations reflected changes in the ratios between different pigments, and that these ratio differences were caused by light conditions.

The action spectrum of photooxidation could be determined only with difficulty. The curve presented in Figure 8 is redrawn from a recording with the field instrument. Correction has been made for a biased baseline, presumably due to different effects of radiations in different spectral regions. The action spectrum of this process must therefore be considered highly approximate. It is, however, evident that blue and red light are more effective than wave-lengths in between. It seems that the photosynthetic apparatus is the quanta-absorbing system.

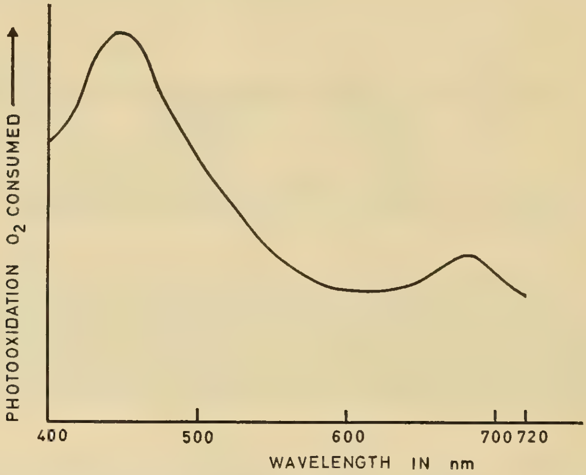

FIGURE 8. "Action spectrum of photooxidation" for the Siphonales *Ostreobium* isolated from within the coral *Favia.*

In vivo absorption measurements. During action spectra analyses of photosynthesis in *Ostreobium* it was noted that the magnitude of the shoulder at 720 nm showed variations, and it was assumed that these changes were caused by differences in light conditions. To test this hypothesis, several *Favia* specimens containing the green layer *Ostreobium* were cut. One-half of each was exposed to shaded sunlight in deck tanks with running sea water. The other half was wrapped in black plastic and placed under an inverted bucket in the same tank. After three days tangential sections were cut out with a band saw from the dead part of the coral containng the green layer *Ostreobium.* These pieces were further trimmed down to about 2 mm, in thickness by grinding, and the final samples ready for spectrophotometric analyses had sizes around 20 × 20 × 2 nm. The absorption spectra of paired pieces, taken from the same coral specimen but exposed to different light conditions (dim light *versus* darkness) for three days, were then compared in a Shimadzu multipurpose MPS-50 spectrophotometer available on board the Alpha

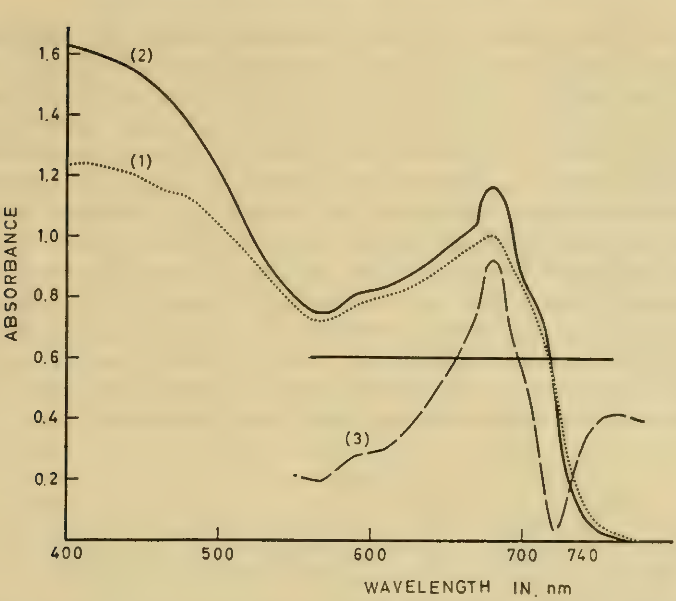

FIGURE 9. *In vivo* absorption measurements of the green layer alga *Ostreobium* isolated from within the massive coral *Favia.* (1) : sample kept in darkness for three days after the cut. (2) : sample exposed to shaded sunlight in sea-water tank for three days after cut. (3) : difference spectrum where (2) is in the sample compartment and (1) used as a reference.

Helix. The results are given in Figure 9. The *in vivo* absorption spectra of two paired samples are given (/1/ and /2/), and also the difference spectrum between these two, where the one kept in light was placed in the sample compartment, and the one kept in darkness as a reference (Fig. 9/31). In the absorption spectra a distinct inflexion at 710–720 nm is visible for both samples. The difference spectrum, however, clearly demonstrates that the sample kept in dim light has

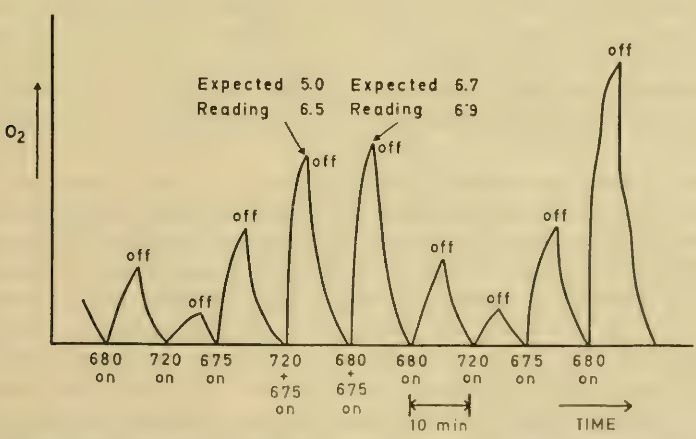

FIGURE 10. Enhancement studies in the green layer alga *Ostreobium.* For explanation see text.

relatively more of pigment(s) absorbing maximally at 680 nm, while the one kept in the dark is richer in pigment(s) absorbing maximally at 720 nm.

Enhancement studies. Such analyses were performed at 675, 680 and 720 nm with the Bausch and Lomb monochromator and the field instrument. Results from one run are shown in Figure 10, where a sample was given one-minute exposure to one of the above wave-lengths, or combinations of these. In the dark the system was then allowed to decrease to the same pO_2 as before the first exposure. In this series the 680 nm irradiation gave a 2.6 cm. reading on the recorder, 720 nm alone gave 1.0, and 675 4.0 cm. The combined effect of 675 and 720 nm at the intensities selected should then give an expected reading of 5.0 with no enhancement. The reading of this combination was 6.5. The next combination (675 and 680 nm) gave 6.9 against expected 6.7, which demonstrates no or insignificant enhancement. To check for the stability of the system, and that pO_2 was below saturation level, the remaining four exposures were performed.

DISCUSSION

The tissues of the massive coral *Favia* were dark chocolate brown due to the pigments of the intracellular symbiotic zooxanthellae. This alga is, particularly during low tide when direct sunlight may hit the coral, exposed to very high light intensities. As expected, intensity/response curves also showed that the dinoflagellate reached saturation at very high light levels, and that no decrease in photosynthetic rate could be achieved in monochromatic light with the equipment used during the investigation.

The action spectrum of photosynthesis in the dinoflagellate is also mainly in accordance with expectation (Haxo, 1960). It responded to radiation in the spectral region between 300 and 720 nm. Peaks and shoulders corresponding to chlorophyll *a* and *c*, and the carotenoid peridinin were resolved, and a distinct peak occurred around 540 nm. The action spectrum of fluorescence excitation as well as the *in vivo* absorption spectrum showed peaks in this spectral region (Shibata, unpublished data). Halldal (1964) observed near-ultraviolet bands in the action spectrum of photosynthesis of *Ulva,* and he suggested that they may correspond to absorption bands of carotenoid(s) being transferred into their cis-form(s) upon excitation. In a similar way the peak at 540 nm in the action spectrum curve of the zooxanthellae coincides well with a plausible position of a cis-form absorption band of peridinin. Experiments to test these suggestions have, however, not been performed.

The dense brown zooxanthellae-cover greatly influences the light conditions inside the coral. Taking into consideration the extremely low light level that is available for the green layer Siphonales, *Ostreobium,* it at first seemed impossible to visualize how this should suffice to drive the photosynthetic apparatus. More than 99.9% of the light is absorbed by the living part of the coral and the zooxanthellae. This 0.1% left is further reduced when passing through the dead part to reach the green layer, and within the green layer the light intensity is estimated to be only 0.0001–0.000001% between 400 and 680 nm and 0.001% at 700 nm of that outside the coral (Shibata, unpublished data). In order to get an idea of the absolute light levels within *Favia,* one may calculate from the most favorable light conditions of 100,000 lux during midday direct sunlight under tropical conditions.

According to this around 100 to 150 lux should prevail at the top of the green layer under the most favorable conditions, usually much less and only a fraction of a lux in the middle or below it (Fig. 1). The intensity/response curves measured clearly demonstrated that *Ostreobium,* developed under the light condition that exists within the massive coral, indeed was sensitive and vulnerable to light. It showed a clear response to 2 erg. $cm.^{-2}$ sec^{-1}, and some samples were even photooxidized at this intensity. Samples which responded with photosynthesis to radiation reached saturation at very low light levels, and were easily photooxidized at somewhat higher light levels. Unfortunately, due to the lack of time, the intensity/response curves were measured only in blue light. *Ostreobium* may tolerate, and more effectively utilize, higher intensity red and far red light, as was suggested in the action spectrum of photooxidation (Fig. 8), where blue light definitely seems to be more effective than longer wave-lengths.

Also the action spectrum of photosynthesis demonstrates that *Ostreobium* favorably utilizes far red light, and that its pigment composition is thus well suited for the light conditions within the coral. The pigment which shows an absorption maximum at 720 nm, and which presumably is a chlorophyll *a* form, is an adaptation to capture the light which penetrates the living coral and the zooxanthellae. About 5% of light at 720 nm penetrates this part, and at the surface of the green layer up to 2% may be left (Fig. 1). The results from enhancement studies also demonstrate that quanta absorbed in the shorter wave-length region may cooperate with quanta absorbed in far red. As a consequence of this, the chlorophyll *a* form with peak at 720 nm should be part of a two-pigment system.

A number of different *in vivo* chlorophyll *a* forms are now known to be present. Krasnovsky and Kosobutskaja (1955) observed spectral shifts in the red chlorophyll *a* absorption peak of living plants. Shibata (1957) in his spectrophotometric studies of protochlorophyll(ide)-chlorophyll(ide) transformations demonstrated that pronounced spectral shifts occurred around 670 to 684 nm. In fully developed plants, Halldal (1958b) directly observed two chlorophyll *a* forms in the blue green alga *Anacystis,* and French (1959) using a derivative spectrophotometer showed the presence of different forms in *Chlorella* and *Euglena.* In the green alga *Ostreobium* at least two such forms are present as can directly be observed in spectrophotometric analyses. One of these forms has a far red absorption peak at 720 nm (Fig. 9). The peak at 680 nm may represent more than one form.

The pigment ratio C_a680/C_a720 evidently could be altered through changes in light conditions. Also Krasnovsky and Kosobutskaja (1955) demonstrated related shifts introduced by light, and Halldal (1958b) showed that alterations in the pigment ratio C_a670/C_a682 were introduced by light and temperature conditions. Thus the existence of different *in vivo* forms of chlorophyll *a* cannot be questioned. These spectral shifts presumably are due to different associations of chlorophyll *a* to macromolecules. It is also evident that the ratios of these different pigment forms may be altered through environmental factors.

One may be tempted to assume that the spectral energy distribution that exists within the massive coral *Favia* is the main cause for the development of the particular chlorophyll *a* form with maximum absorption around 720 nm. The far red light that predominates within the coral should, according to this assumption, stimulate the formation of the far red-absorbing C_a720. Comparative analyses performed with cut *Favia* specimens also showed that "white" light increased the ratio C_a680/C_a720, thus supporting such a view.

It should, however, be kept in mind that both light quality and light intensity have been demonstrated to introduce pigment ratio variations in red and blue-green algae. Brody and Emerson (1959) studied the effect of pigments formed in the red alga *Porphyridium* in different parts of the spectrum. They concluded that alterations in pigment composition were mainly an adaptation to adjust the photosynthetic rate in order to make the quanta absorbed by the pigment system fit succeeding enzymatic processes. According to this view the so-called complementary chromatic adaptation should function only at low light levels. Halldal (1958b) observed a similar system in pigment adaptation of blue-green algae in "white" light, where low light intensities resulted in much higher ratios of accessory pigments/chlorophyll *a* than in bright light.

Taking into consideration the results from the above mentioned analyses on pigment ratio changes, where both the intensity of colored and "white" light were decisive, one should be somewhat cautious in interpreting results from such experiments.

A light quality adaptation aimed to capture far red light seems to be the reasonable explanation for the high ratio C_a720/C_a680 found in *Ostreobium* grown inside the massive coral *Favia*. On the other hand the development (enrichment?) of the chlorophyll *a* 720 form may also be considered as an adaptation to capture more light in general by an expansion of the absorption toward longer wave-lengths. The first mentioned alternative certainly is the more attractive of the two, but direct experimental analyses are needed to give the answer.

Some difficulties exist to make pigment composition of *Ostreobium* fit the concept of the now commonly accepted two-pigment system in photosynthesis. This briefly states that light quanta absorbed by C_a670 start an electron flow that is supplied with more energy through quanta absorbed by the chlorophyll *a* form C_a682. The chlorophyll *a* form C_a705 or P700, which was observed by Kok and Hoch (1961) to undergo absorption changes upon illumination, is in accordance with the two-pigment system assumed to function as an energy sink. The Kok-pigment, C_a705, does not fit the widely accepted pattern of electron flow through a two-pigment system in *Ostreobium,* as it absorbs at shorter wave-lengths than C_a720. Energy transfer form C_a720 to C_a705 is thus excluded. One possibility that could exist and satisfy the requirement for a two-pigment system in *Ostreobium* involving two quanta-absorbing pigments, C_a680 and C_a720, is that C_a705 has been substituted by another chlorophyll *a* form which absorbs at longer wavelengths than C_a720. Absorption spectra measurements indicating the presence of presumably chlorophyll forms with absorption peaks around 740–750 nm have been reported (Lippincott *et al.,* 1962; Aghion, 1963; Govindjee *et al.,* 1961; Govindjee, 1963).

This investigation was performed in cooperation with Drs. F. T. Haxo, S. Jeffrey and K. Shibata on board the R/V Alpha Helix of Scripps Institution of Oceanography on an expedition to the Great Barrier Reef off Australia, August–October, 1966. I would like to express my sincere thanks for the opportunity to participate in this program. The corals used were identified by Dr. E. C. Allison of San Diego State College, and the green layer algae by Dr. M. Nizammudin, University of Pakistan. Thanks are due to Dr. Keith Boardman (CSIRO, Canberra) for the loan of the Texas Instrument Co. recorder used during this investigation.

SUMMARY

1. The living part of the massive coral *Favia* is inhabited by a symbiotic dinoflagellate (Zooxanthellae) which resembles *Symbiodinium microadriaticum*. Intensity/response curves for this alga show that saturation occurred at 95,000 erg. cm.$^{-2}$ sec^{-1} in blue light (440 nm). Action spectrum determinations were performed either with an automatic field instrument, or by a point method. The results are mainly as to be expected for a dinoflagellate. A high activity band was noticeable around 540 nm. A similar band was also visible in the *in vivo* excitation spectrum of fluorescence, as well as in the *in vivo* absorption spectrum (Shibata, unpublished data).

2. A green alga *Ostreobium* (Siphonales) can live and photosynthesize within the massive coral. The light levels within the coral are very low; however, wavelengths above 700 nm are preferentially transmitted by the living part of the coral with the zooxanthellae. *Ostreobium* showed saturation at or below 1000 erg. cm.$^{-2}$ sec^{-1} at 440 nm. Some samples showed photooxidation at all light intensities. It was shown that the position of *Ostreobium* within the coral was decisive for its response to light. Action spectra determinations and *in vivo* spectrophotometric analyses showed that *Ostreobium* was adapted to the particular light conditions within *Favia* in that a pigment, presumably a chlorophyll *a* form with absorption maximum at 720 nm (C_a720) actively participated in photosynthesis. The effect at 720 nm was up to 80% of the effect at 680. Enhancement studies at 675 and 720 nm gave positive results.

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CHEMICAL ANALYSIS OF GLYCOPROTEINS IN THE EGG SURFACE OF THE SEA URCHIN, ARBACIA PUNCTULATA[1]

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Since Runnström (1930) reported that sea urchin eggs produce acid at the time of fertilization, a number of studies have employed various methods to determine the source of this acid (Monné and Hårde, 1951; Runnström and Immers, 1956; Immers, 1961a, b; Mehl and Swann, 1961; Aketa, 1962; Fujii and Ohnishi, 1962). The present author found that at fertilization, jelly-free eggs of Japanese sea urchins extrude protein-containing acid polysaccharides different in chemical composition from the jelly coat (Vasseur, 1947; Tyler, 1948). He suggested that this fertilization product might be the so-called fertilization acid (Ishihara, 1964), and tested this possibility using the eggs of a Japanese sea urchin (1968). The production of similar substances has been confirmed in *Arbacia punctulata* by Gregg and Metz (1966) and Metz (1967), who report the fertilization product to be a complex mixture which acts as a fertilizin and contains some components immunologically the same as jelly fertilizin.

The present work presents evidence that the fertilization product is a substantial source of fertilization acid in *Arbacia punctulata,* and describes some of its physical and chemical properties, including a comparison with those of the jelly coat substance.

MATERIAL AND METHODS

The sea urchin, *Arbacia punctulata,* used as material was supplied by courtesy of the Marine Biological Laboratory at Woods Hole, Mass., in the summer of 1965. The jelly coat substance and fertilization product were obtained as previously described (Ishihara, 1964, 1968). Each volume of eggs was measured after the egg jelly had swollen and the eggs settled to the bottom of the beaker. The volume of jelly-free eggs corresponded to about one-fifth of the volume of intact eggs. After repeated dialysis against distilled water using Visking tubing (size, 23/32), the lyophilized substances were brought to Japan for analysis.

Ultracentrifugation was performed with a Beckman Model E Analytical Ultracentrifuge, using 0.075, 0.10, 0.15, 0.20% solutions of jelly coat substance in pH 9.0 borate buffer ($\mu = 0.1$). Amino acids were analzed with a Yanagimoto Type LC-5 Automatic Amino Acid Multi-analyzer, after hydrolysis with 5.7 N HCl at 120° C. for 8 hours. The determination of amino-terminal amino acid was carried out by the dinitrophenylation procedure. Dinitrophenylamino acids used as standards were purchased from Wako Pure Chemical Industries, Ltd., Osaka,

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[1] Dedicated to Professor Juro Ishida on the occasion of his sixtieth birthday.

Japan. For the determination of hexose, the hydrolysate obtained with 1 N H_2SO_4 at 105° C. for 4 hours (Ishihara and Schmid, 1967) was desalted with Dowex 50-X 12 (H form) and Amberlite IR-45 (carbonate form) after neutralization with saturated $Ba(OH)_2$. Hexoses were separated by paper chromatography, using benzene-n butanol-pyridine-water (1:5:3:3 V/V) as the developing irrigant, and the ratio of individual hexoses was determined after the method of Whistler and BeMiller (1962). The anthrone reaction was employed for the final determination of neutral hexose in the materials, using as standard a mixture of authentic hexoses in the proportion determined by the procedure mentioned above. The amount of fucose was also determined by the cysteine reaction, after the method described by Winzler (1955). Hexosamine was determined according to the method of Elson-Morgan described also by Winzler (1955). For the sulfate determination, the hydrolysate obtained with 4 N HCl at 105° C. for 4 hours was evaporated with a rotary evaporator to remove completely the hydrogen chloride, and then the liberated sulfate was estimated after the method of Iwasaki *et al.* (1957). Cations were analyzed on unhydrolyzed material dissolved in deionized water by means of a Hitachi Model FPF-2 Flame Photometer. The infrared spectrum was analyzed in the range from 4000 to 400 cm.$^{-1}$ in wave-number (2.5 to 25 μ in wave-length) with a JASCO IR-G Infrared Spectrophotometer, using the KBr tablet method, and corrected at 2924, 1603 and 906 cm.$^{-1}$ employing polystyrene film. The abbreviations used below are: FP for the fertilization product which diffuses out of the elevating fertilization membranes of jelly-free normal eggs, and TFP for the fertilization product obtained from eggs of which the vitelline membrane was removed by treatment with trypsin after removal of the jelly coat.

TABLE I

Yields of jelly substance and fertilization product from eggs of Arbacia punctulata

Products	Egg volume (ml.)	Dry weight of products (mg.)
Jelly	800	288.7
TFP	400	60.2
FP	400	18.8

RESULTS

Yields of jelly substance, FP and TFP

As shown in Table I, the yield of non-dialyzable jelly substance was about 2.5 times greater than the amount of TFP and about 8 times that of FP. The higher yield of TFP is expected since it must contain the perivitelline space substance and vitelline membrane-hardening substance in addition to FP. Ultracentrifugation showed that the jelly substance obtained in this manner was practically homogeneous; the sedimentation coefficient (Fig. 1) estimated in various concentrations at constant temperature of 21° C. and at 59,780 r.p.m. was 7S (S = Svedberg unit). The reciprocals of the S values were extrapolated to obtain a clear point.

The sedimentation velocity of FP was not analyzed because the chemical analysis of this substance revealed it to be a mixture.

Chemical analysis

The amino acid content was found to represent 14.3, 78.6 and 44.7% in dry weight of the jelly, TFP and FP, respectively (Table II). Jelly substance, TFP and FP differ greatly among each other in their total contents of amino acids, but they contain the same amino acids in approximately the same proportions. Aspartic and glutamic acids are predominant in both TFP and FP, whereas there is relatively more isoleucine, leucine and phenylalanine in the jelly substance. There is less proline, glycine, alanine, cysteine, tyrosine and arginine in the jelly than in TFP and FP. The large amounts of cysteine or cystine suggest that the constituent proteins have a complex structure, especially in TFP whose cystine was not completely cleaved even under a degree of hydrolysis which destroyed hexosamine. The N-terminal amino acids of the three substances are listed in molar ratio in Table III. This table shows that the amino acid-containing constituents of TFP and FP are mixtures of polypeptides or proteins. The N-terminal amino acids of jelly substance consist of two moles of phenylalanine and one mole of arginine; no minor components could be detected. This result suggests that the jelly is composed of three polypeptide chains.

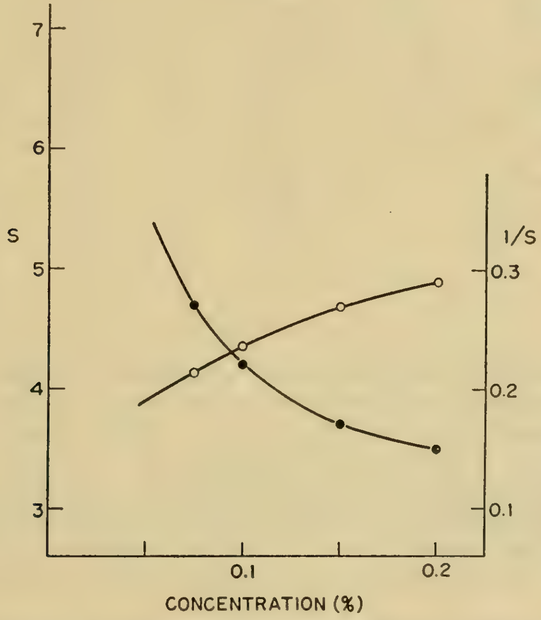

FIGURE 1. Sedimentation coefficient of jelly coat substance. Solid circles: Svedberg unit (S) plotted against concentration of jelly. Open circles: Reciprocal of S values plotted against concentration of jelly. Conditions, see text.

TABLE II

Amino acid compositions of jelly and fertilization product
(in percentages of total dry weight)

Amino acid	Jelly	TFP	FP
Aspartic acid	1.65	12.5	7.05
Threonine	1.30	6.05	4.04
Serine	1.09	5.13	2.85
Glutamic acid	1.66	12.0	5.50
Proline	0.55	5.48	3.10
Glycine	0.68	4.83	3.82
Alanine	0.83	3.41	1.95
Cysteic acid	0.25	3.24	2.74
Cystine	—	1.50	—
Valine	1.23	4.76	2.45
Methionine	0.08	0.25	—
Isoleucine	0.93	3.23	1.67
Leucine	1.11	4.36	2.28
Tyrosine	0.55	3.12	1.18
Phenylalanine	1.16	3.73	1.78
Lysine	0.54	1.27	1.15
Histidine	0.33	1.01	0.82
Arginine	0.36	2.74	2.35
Total	14.3	78.6	44.7

The results of the chemical analyses are summarized in Table IV. In addition to the difference in amino acid content, there are marked differences in hexose and hexosamine contents between the jolly and FP or TFP. The jelly contains fucose as a major component and mannose and galactose as minor components. The jelly mannose was not detected by staining with aniline phthalate but was revealed by ammoniacal silver nitrate. TFP contains fucose, mannose, glucose and galactose approximately in the ratio of 2:1:1:2, which was estimated by quantitative paper chromatography. The presence of such sugars in FP was also reported by Gregg

TABLE III

Amino-terminal amino acids of jelly and fertilization product
(moles per mole of substances)*

Amino acid	Jelly	TFP	FP
Aspartic acid	—	1.9	1.4
Glutamic acid	—	4.7	0.98
Threonine	—	0.91	0.23
Serine	—	1.5	0.20
Alanine	—	0.79	0.11
Valine	—	0.97	—
Leucine	—	0.57	—
Glycine	—	0.29	—
Phenylalanine	1.8	0.33	0.94
Arginine	1.0	1.3	0.85

* Molecular weight of substance is taken as 300,000 (Tyler *et al.*, 1954).

and Metz (1966). A small but significant amount of hexosamine was found in the jelly and in the proportion of one to one mole of neutral hexose in FP and TFP. In all three substances, the sulfate-to-total hexose ratio is approximately 1:2 in percentage; *i.e.*, a molar ratio of 1:1, as in other sea urchins (Vasseur, 1947; Ishihara, 1964). Inorganic cations were determined for Ca^{++}, Na^{+} and K^{+} of the jelly and TFP as shown in Table IV. The amounts of cations in jelly substance are small in comparison with that of sulfate, whereas there are enough cations in TFP to mask the sulfate groups in such forms as $—OSO_3—Na$ and $2—OSO_3{=}Ca$, as observed in the FP of other sea urchins (Ishihara, 1968).

TABLE IV

Chemical compositions of jelly and fertilization product
(in percentages of total dry weight)

Constituent	Jelly	TFP	FP
Amino acid	14.3	78.6	44.7
Hexose	37.8	9.7	10.1
Fucose	36.5	3.4	3.2
Mannose	+*	1.5	+
Glucose	0	1.6	+
Galactose	1.3	3.2	+
Hexosamine	2.6	9.2	10.0
SO_4^{--}	19.0	5.0	6.4
Inorganic cations	1.7	1.3	–**
Ca^{++}	0.6	0.7	–
Na^{+}	0.8	0.4	–
K^{+}	0.3	0.2	–
Total	75.4	103.8	71.2

*+: detected by paper chromatography but not estimated quantitatively. **–: not determined.

Infrared spectrum

In both materials (Fig. 2), strong stretching vibration due to hydroxy groups in the range from 3800 to 3200 cm.$^{-1}$ and a medium absorption spectrum produced by the bending vibration of C—H at 2950 cm.$^{-1}$ were observed; these are characteristics of carbohydrates. Particularly with jelly substance, there occurred a characteristic absorption spectrum at 970 cm.$^{-1}$ arising from methylpentose. The maximum stretching vibrations of C—O were detected at 1168, 1085, 1055, 1025 cm.$^{-1}$ in jelly and 1045 cm.$^{-1}$ in TFP. The simple absorption curve in this range of the latter suggests that it includes a substance of high molecular weight. The presence of —NH—CO— groups in both substances is suggested by the bending vibration of N—H at 1540 cm.$^{-1}$ and the stretching vibration of C=O in a monosubstituted acetoamide group near 1640 cm.$^{-1}$, both of which are presumably due to peptide bond or N-acetylhexosamine. It is obvious that there are esterified sulfates in both jelly and FP, as shown by the stretching vibration of S—O at 1240 cm.$^{-1}$. The absorption spectrum at 840 cm.$^{-1}$ due to C—O—S binding in jelly provides evidence for assigning to the sulfate ester an axial location on the sugar molecule. In other words, the sulfate ester is located at the C-2 or C-3 position of fucose. In TFP, however, no special absorption in the lower wavenumber field was detected.

Discussion

Although the method used in this study for isolating the jelly substance of *Arbacia* eggs was different from that of Tyler (1949), the yield was about the same. Analysis by ultracentrifugation indicates that the jelly substance obtained is identical with the fertilizin prepared by Tyler *et al.* (1954) and Tyler (1956), although the biological activity was not studied since living material is not available in Japan. The amino acid composition of *Arbacia* jelly closely resembles that of *Lytechinus pictus* (Tyler and Tyler, 1966).

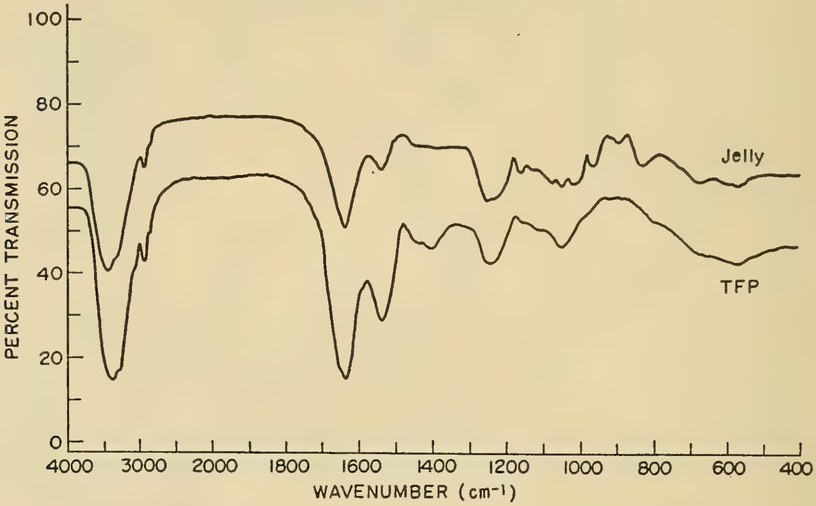

Figure 2. Infrared spectra of jelly coat substance and TFP.

The larger amount of amino acid in TFP than in FP is probably due to the protein or proteins making up the membrane-hardening substance, as is also suggested by the high N-terminal amino acid content of TFP. In other words, the vitelline membrane-hardening substance is protein-rich and present in relatively small amount. It is highly probable that the fertilization product is extruded through the elevating vitelline membrane immediately after fertilization before it hardens completely, and that the major part of this substance remains in the perivitelline space. It should probably be considered that the acid treatment used to remove the jelly coat has affected the vitelline membrane to some extent. The heterogeneity of FP was also demonstrated immunologically by Gregg and Metz (1966) and Metz (1967).

In analyzing for N-terminal amino acid, three moles of amino acid were detected in the jelly, suggesting that it is composed of three polypeptide chains. When these analyses were repeated later, however, larger amounts of N-terminal amino acids were found in the jelly substance. The possibility that this increase is due to decomposition of the jelly protein is being investigated by studies on the effect of aging, dialysis and ultraviolet irradiation which have similar effects on the agglutinating activity of the jelly.

It is widely recognized that the acidic character of the jelly substance is due to the high content of sulfate groups (Vasseur, 1947, 1948; Tyler, 1948, 1949; Runnström and Immers, 1956; Minganti and Vasseur, 1959). Bound sulfate is present in a relatively high amount in the sea urchin egg jelly studied here. However, one-third of the hydrolyzable sulfate (4 N HCl) is very labile, as shown by the fact it is liberated under the condition of hydrolysis with 0.04 N HCl at 100° C. for 4 hours, as pointed out earlier by Vasseur (1948). Infrared spectrum analysis of the jelly indicates that sulfate is bound to fucose with an ester linkage in the position of C-2 or C-3. According to Vasseur (1952), the C-3 position of fucose in the jelly of *Echinocardium cordatum* is free. This makes it likely that the sulfate group of *Arbacia* jelly, as well as that of *E. cordatum,* is linked to the C-2 position of fucose. Esterified sulfate was also detected in the FP, but the position of linkage is not obvious.

Cations such as Ca^{++}, Na^{+} and K^{+} are present in both jelly and FP. That the FP is still acidic even in the presence of significant amounts of cations indicates that the inorganic cation-free FP is a strong acid which links with cations from sea water when the cortical granules open (Ishihara, 1968). In the case of jelly coat substance, the importance of cations in fertilization has been emphasized by Monroy *et al.* (1954).

The percentage totals of the substances detected by the various methods employed in this study are lower than would be expected, since the values were not corrected for water added in hydrolysis. Only 75.4% of the jelly constituents were accounted for in the analysis; the nature of the remainder is unknown. According to Isaka, jelly contains a thiobarbiturate-sensitive, sialic acid-like substance which is very labile, and is liberated by mild acid treatment (personal communication). Carboxylic acids such as uronic and sialic acids have not been detected in the jelly substance by infrared analysis and have not been found in the jelly of other sea urchins (Ishihara, 1964) or of the starfish *Asterias amurensis* (Muramatsu, 1965). Since the jelly studied here was collected by treatment with acidified sea water (pH 4.5) followed by drastic dialysis, it is likely that any sialic acid present was removed during preparation.

FP was also found to include unknown constituents. Isaka found the presence of a bound sialic acid-like substance also in FP of Japanese sea urchins (personal communication). It seems to be important also to test for other components, such as lipids, which were not studied here.

I wish to thank the Woods Hole Marine Biological Laboratory for the supply of sea urchins; Dr. Shinya Inoué, for the use of his laboratory space; and Dr. Karl Schmid at Boston University School of Medicine for the facilities provided for the purification of the collected material. I am indebted to Mrs. Masako Kikuchi at the University of Tokyo for the ultracentrifugation analysis, to Mr. Nobuaki Abe at the Research Laboratory, Snow Brand Milk Products Co., Ltd. for the amino acid analysis, to Dr. Masaakira Maeda of our laboratory for the infrared analysis and to Dr. Jean C. Dan at Ochanomizu University for reading the manuscript and her kind advice.

SUMMARY

The production of an acid glycoprotein at fertilization was confirmed to occur in the egg of *Arbacia punctulata*. Chemical analyses of such "fertilization product" and jelly coat substances were carried out. These substances are both glycoproteins containing hexose, hexosamine, sulfate, inorganic cations and 17 amino acids. However, the differences in the respective amounts of their constituents show that the fertilization product, which is believed to be the perivitelline space material, is a different substance from jelly. The release of this substance is detected as the formation of "fertilization acid." The results of the analyses also suggest that the membrane-hardening substance is a protein or protein-rich substance.

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STUDIES ON CARDIOVASCULAR PHYSIOLOGY OF TUNICATES

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The tubular tunicate heart is unique within the chordates because pacemakers at each end alternate in periods of activity and change the direction of contraction. Peristaltic waves can force the blood through the circulatory system in either direction because there are no structural valves.

Many mechanisms of reversal have been suggested but "back-pressure" and "pacemaker fatigue" theories have had the greatest support. In a back-pressure mechanism, the arterial blood pressure increases above the venous pressure during a series of contractions in one direction which differentially changes the excitability of the two pacemakers and results in a reversal (Lahille, 1890; v. Brücke, 1925). However, isolated hearts may regularly reverse, suggesting that changes in blood pressure are not necessary for reversal; and, when hearts are cut in two each half may show periods of activity and rest, suggesting pacemaker fatigue (Krijgsman, 1956).

A comparative approach using both isolated and hearts *in situ* of four genera was used in this investigation because many published observations that are contradictory are from different genera. Thus, many previously reported experiments concerned with pacemaker activity in half-heart preparations, with the effect of heating or cooling a pacemaker region and with the effect of electrical stimulation of the heart have been repeated and extended. New techniques of electrically and mechanically recording the heart activity have been developed so that a more direct analysis of heart function has been possible. In view of the new experiments presented here, many older observations are current. This approach permits a critical interpretation of many previous investigations and an evaluation of the proposed mechanisms of reversal.

The mechanics of diastolic filling and the formation of the peristaltic wave are described and their importance to the general performance of the heart and beat reversal has been assessed.

METHODS

The records presented are from adult *Ciona intestinalis* (body length 6–10 cm.) collected at the Marine Biological Laboratory, Woods Hole, Massachusetts. For comparative purposes, hearts of adult *Corella willmariana, Ascidia callosa, Chelyosoma productum* and *Ciona intestinalis* (the latter shipped from Cali-

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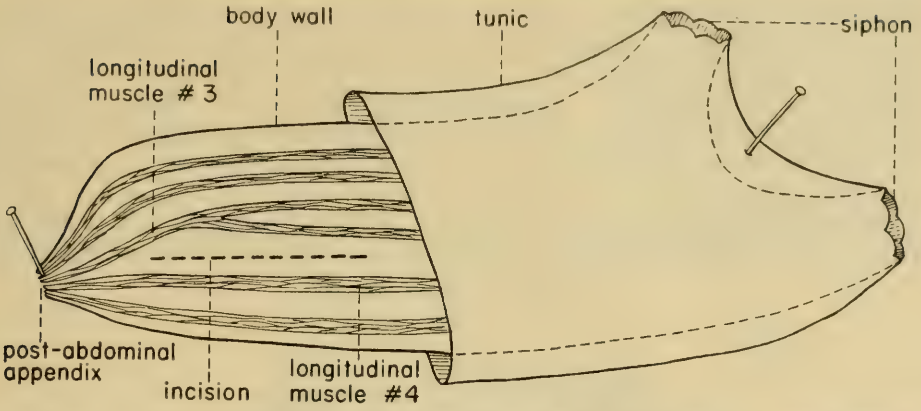
body wall
tunic
siphon
longitudinal muscle # 3
post-abdominal appendix
incision
longitudinal muscle #4

FIGURE 1. Steps for the dissection of the *Ciona* heart. The animal is viewed from the right side. Step 1. The posterior half of the tunic has been removed, exposing the body wall. After the animal had expanded and begun siphoning, the post-abdominal appendix and tunic between the siphons was pinned to a cork. Step 2. A 2-cm. incision was quickly made between the longitudinal muscle bands No. 3 and No. 4. The heart and visceral organs were then expelled from the coelom due to muscular contractions of the body wall. Step 3. The animal was allowed to relax before the epicardiac cavities were opened to expose the pericardium as shown in Figure 2.

fornia) were studied at the Friday Harbor Laboratories, San Juan Island, Washington.

Detailed methods for preparing either an isolated heart or exposing a *Ciona* heart *in situ* are described by v. Skramlik (1926a) and Kriebel (1964). The posterior half of the tunic was removed and a 2-cm. incision was made on the right side over the visceral region through the body wall between the 3rd and

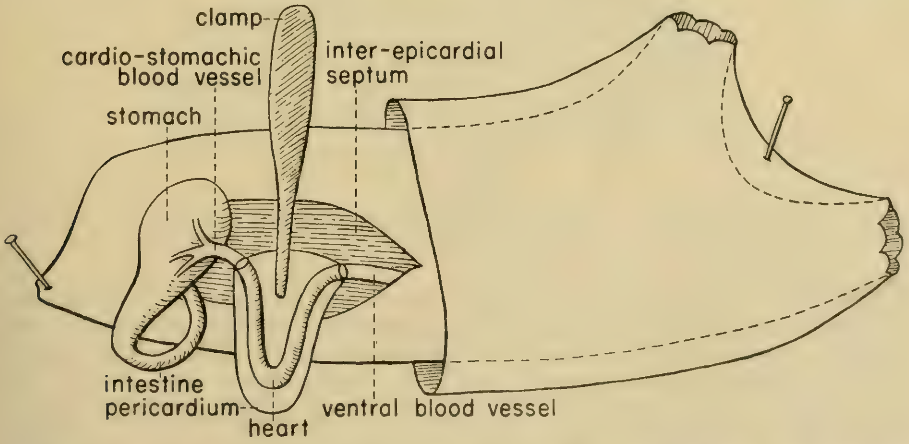
clamp
cardio-stomachic blood vessel
stomach
inter-epicardial septum
intestine
pericardium
heart
ventral blood vessel

FIGURE 2. Exposed heart preparation. The pericardium has been clamped so that the heart is supported and the major blood vessels have been left intact so that the blood circulates in a nearly normal manner. The ventral and cardio-stomachic blood vessels were either ligated or cannulated before freeing the heart from the blood vessels.

4th longitudinal muscle bands (Fig. 1). Such animals lived 3–5 days in sea water maintained at 10–20° C. Before the epicardiac membranes were severed, freeing the pericardium, small blood vessels in the interepicardiac septum were pinched with forceps to prevent bleeding. The pericardium was clamped between the arms of the V-shaped heart (Fig. 2). This method of holding the pericardium did not seem to impair the action of the heart, as blood continued to circulate for up to two days.

Isolated hearts were prepared either by ligating and cutting the ventral and cardio-stomachic blood vessels or cannulating the vessels (Fig. 3). The cannulae were connected to reservoirs filled with sea water to the same level as in the heart chamber. A valve arrangement permitted independent changes in applied pressure in each arm of the heart upon the raising or lowering of the reservoirs.

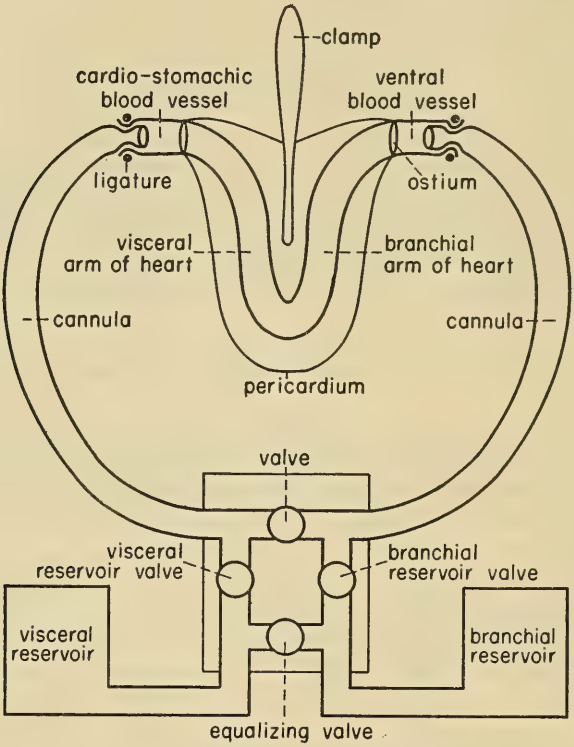
clamp
cardio-stomachic blood vessel
ventral blood vessel
ligature
ostium
visceral arm of heart
branchial arm of heart
cannula
cannula
pericardium
valve
visceral reservoir valve
branchial reservoir valve
visceral reservoir
branchial reservoir
equalizing valve

FIGURE 3. Cannulated heart. The valve arrangement permitted increases in water pressure in either arm of the heart.

Mechanical records of isolated hearts were made with a Grass FT-03 strain gauge positioned in line with each arm of the V-shaped heart (Fig. 4). Because the pericardium was clamped between the arms of the heart, tension resulting from contraction of one arm was not transmitted by the pericardium to the opposite arm. The two directions of contraction generally produced different signals so the direction of contraction could be ascertained from the records.

Suction electrodes (Florey and Kriebel, 1966) were used for extracellular recording and for stimulation of the heart. The electrode tips were made from polyethylene tubing tapered to a long flexible tip of 50–100 μ diameter. The extracellular signals were amplified by low-level A. C. preamplifiers and recorded on a polygraph. By pulling part of the raphe into the electrode tip, hearts *in situ* were stimulated and recorded from.

The temperature of localized areas was changed by perfusing water of the desired temperature through small thermodes. The thermodes were made by insert-

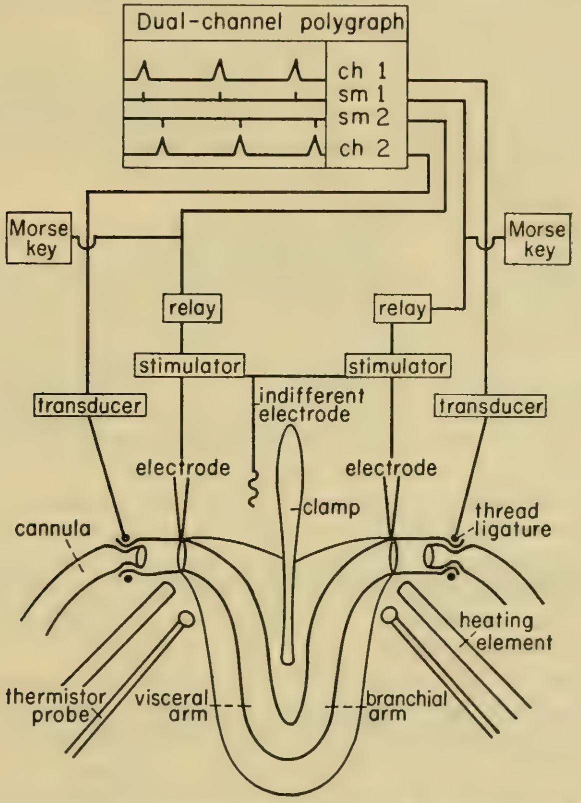
Dual-channel polygraph
ch 1
sm 1
sm 2
ch 2
Morse key
Morse key
relay
relay
stimulator
stimulator
indifferent electrode
transducer
transducer
electrode
electrode
clamp
cannula
thread ligature
heating element
thermistor probe
visceral arm
branchial arm

FIGURE 4. The isolated heart showing the position of the transducers, suction electrodes, heating (or cooling) elements and cannulae. This shows the type of polygraph record obtained during an advisceral pulsation-series. When the waves of contraction started in the branchial arm of the heart, the contractions were first recorded by channel 1 (ch. 1) since the clamp prevented the pull developed by the branchial arm from being transmitted to the opposite transducer. The start of the waves has also been recorded by the signal magnet (s.m. 1), which had been manually triggered by a Morse Key. As the waves of contraction reached the visceral end of the heart, the second signal magnet (s.m. 2) was activated by the second Morse Key. Electrical stimulation or recording was accomplished by drawing part of the cardiovascular junction into a suction electrode. A single indifferent electrode was used to complete the circuit. The pacemaker regions of the heart are located at the ostia. Small heating or cooling elements were positioned as shown to locally change the temperature of the pacemaker region. Temperature was monitored by thermistor probes.

ing a piece of small stainless steel tubing (6 in. long by $\frac{1}{16}$ in. dia.) into a larger one which was sealed with solder at the end. Temperature was monitored with a thermistor.

DEFINITION OF TERMS

V. Skramlik (1926a) demonstrated that the usual pacemaker regions in *Ciona* hearts are well-defined areas at the two ends of the heart. The pacemaker closer to the pharyngeal basket is referred to as the *"branchial" pacemaker;* and the pacemaker closer to the viscera is referred to as the *"visceral" pacemaker.* It is customary to designate contractions originating at the visceral end of the heart as *abvisceral* and contractions starting at the branchial end as *advisceral.*

For convenience, the terms *"paced"* and *"driven"* are used to distinguish contractions initiated by pacemakers from those produced by electrical stimulation.

The time between the start of the last contraction in one direction and the start of the first contraction in the opposite direction is referred to as the *reversal-pause* (Fig. 5, record 1).

The term *ipsilateral* is used to designate the end with the dominant pacemaker, *i.e.,* the arm of the heart in which the wave of contraction originates. Extrasystoles that originate from the active pacemaker are termed *ipsilateral extrasystoles* to distinguish them from those arising in the nonpacing end of the heart which are termed *contralateral extrasystoles.* A *premature systole* originating in the ipsilateral arm may occur before the normal interval between systoles has elapsed with no compensatory pause. A formerly quiescent pacemaker may initiate a contralateral extrasystole (termed an *antiperistaltic twitch,* by Schultze, 1901) which may be followed by a reversal-pause (Fig. 5, record 2).

The period during which one pacemaker is pacing the heart and the subsequent reversal-pause (if present) constitutes a *reversal-period* and it is termed a *pulsation-series.*

Waves of contraction may arise from a formerly quiescent pacemaker giving rise to a reversal by *pacemaker competition* (German, *Wettstreit*). This mode of reversal shows many variations (Fig. 5).

Since the heart wall is very thin (10 μ), it collapses when emptied of blood. Thus the heart can be filled to various degrees. A *normally full* heart refers to the condition in which the heart is just filled with fluid. More fluid stretches the heart wall and in this condition the heart is *overdistended.* For convenience, isolated hearts are said to be empty, one-fourth, one-half, full or overdistended with blood.

RESULTS

A. *Durations of the pulsation-series and the frequency of contractions*

Unless specific mention is made to the species, these results were obtained from *Ciona* hearts. However, the performance of *Corella, Ascidia* and *Chelyosoma* hearts was essentially the same. After opening animals to expose hearts, contractions were irregular and hearts either reversed by pacemaker competition or not at all (in which case the branchial pacemaker remained dominant). However, regular reversals occurred about every 12 min. after animals relaxed and began to siphon and blood flow became nearly normal. After a reversal pause, the newly active pacemaker started slowly, reaching its greatest frequency within a few minutes and then decreasing in frequency until it stopped. The average maximal frequency of 5 *in situ* hearts was 21.3 beats/min. for the branchial pacemaker and 20.0/min. for the visceral pacemaker. Towards the end of pulsation series, the average frequency of both pacemakers fell to 17.0/min. (15° C.). The difference in branchial and visceral pacemaker frequencies assures pacemaker competition reversals since a phase shift would occur.

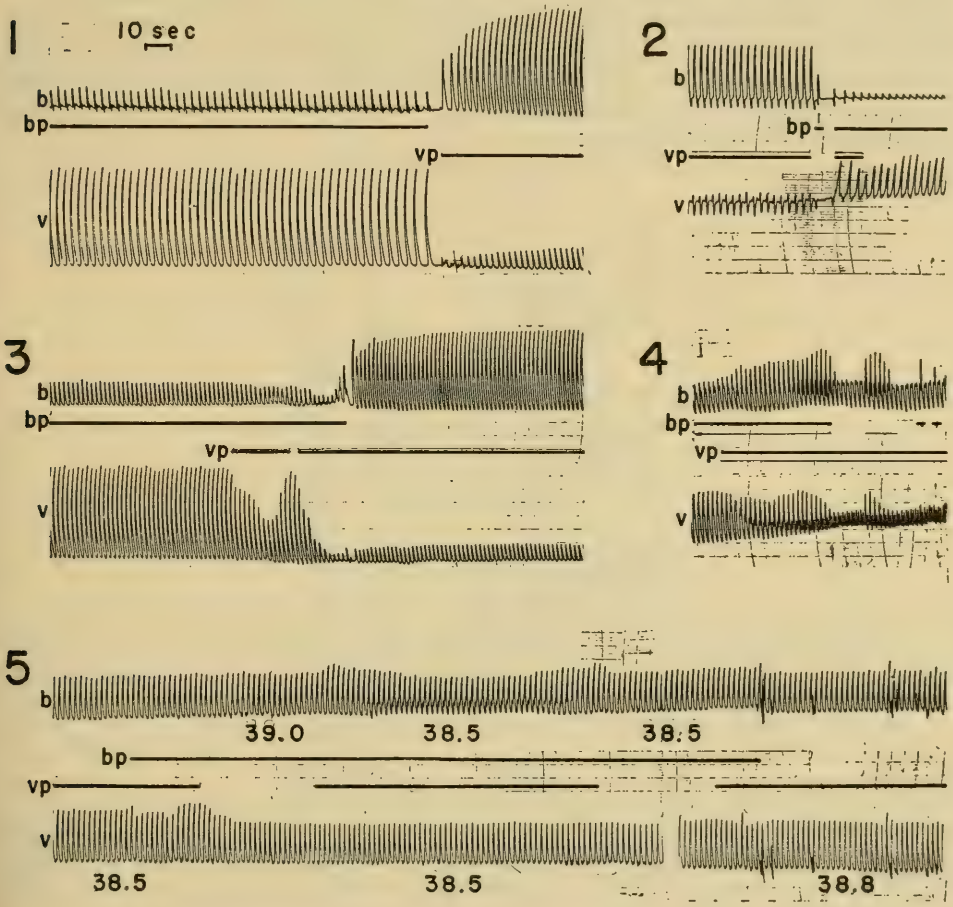

FIGURE 5. Simultaneous mechanical records of contractions from both arms of five different isolated hearts showing modes of reversals. (b) contractions of branchial arm; (v) contractions of the visceral arm; (bp) indicates branchial pacemaker activity; and (vp) indicates visceral pacemaker activity. Numbers below records indicate frequency of contraction per minute during a 20-sec. interval. The usual mode of reversal is shown in record 1 which shows a reversal-pause. Records 2 to 5 show variations in *pacemaker competition* reversals which occurred after a period of time when contraction started at both ends.

When isolated, about 70% of the hearts contracted irregularly at first and some became regular after a period of time. In some hearts a pacemaker (usually the branchial) remained active for several hours without interruption. Reversals occurred irregularly in others and long periods of inactivity (usually 5–20 seconds, but up to 5 min.) occurred between periods of rhythmical activity. In these cases either the formerly dominant pacemaker, or the formerly inactive pacemaker started after the quiescent period. Irregular pacemakers did not slow before stopping. As will be shown in section C, beat irregularity resulted when hearts were overdistended with blood and rhythmical activity was restored by adjusting the blood volume.

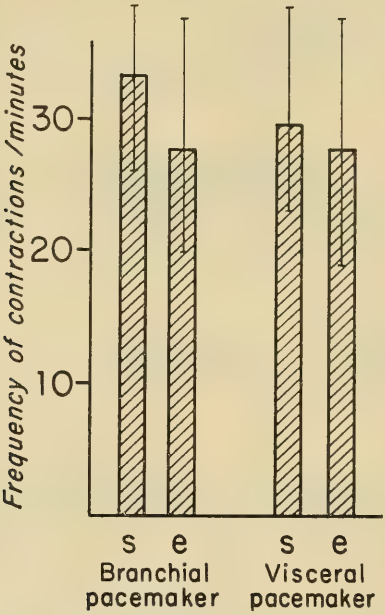

FIGURE 6. The average frequency of 16 isolated hearts at the start and end of branchial and visceral pulsation-series. Abscissa: (s) start of pulsation-series; (e) end of a pulsation-series. The range is represented by the center line within the bar.

The frequencies of 16 isolated hearts showing reversals were analyzed and found to be similar to those recorded while *in situ*. Twelve of the 16 preparations regularly reversed every 10 to 15 minutes and the branchial pacemaker had the longer period of activity. Occasionally, the durations of the pulsation-series in a single heart varied by a factor of two to five, but generally the variation was within 50%. The frequencies of the branchial or visceral pacemakers when compared during the same phase of the pulsation-series in consecutive reversal-periods differed by not more than 10%. The frequency was usually maximal after the first minute and then gradually decreased until a reversal occurred (Fig. 6).

B. *The effect of the removal of the pericardium*

When pericardia were punctured, hearts *in situ* filled until the entire pericardial space was occupied. Thus, the pericardium is relatively nondistensible and prevents overdistention of the heart during diastolic filling.

Hearts were ligated while *in situ* during normal blood flow and then isolated. When the pericardia were pierced, pericardial fluid escaped and the hearts usually reversed and contracted irregularly for a few minutes. Thereafter, the hearts contracted at a decreased frequency (Fig. 7), and reversed more often (Fig. 8). When the pericardium was removed, the frequency of contraction decreased and reversals increased (Figs. 7 and 8) but the general performance was unchanged. The pericardium was left intact unless otherwise stated.

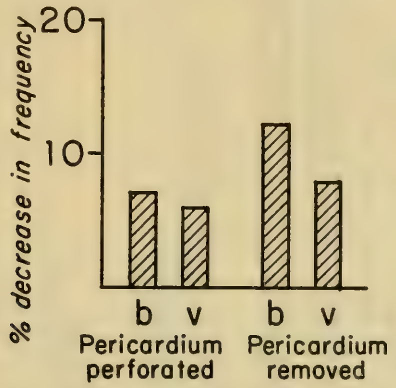

FIGURE 7. The per cent decrease in pacemaker frequency in isolated hearts due to a decrease in pericardial pressure and subsequent removal of the pericardium. Abscissa: (b) Branchial pacemaker; (v) Visceral pacemaker. A perforation was first placed in the pericardium to eliminate the pericardial pressure. Then one surface of the pericardium was removed to expose the heart. The bars represent the average decrease in frequency of 6 branchial and 6 visceral pacemakers.

Hearts gradually swelled when not protected by the nondistensible pericardium. The mode of reversal eventually changed and the duration of the pulsation-series increased until the branchial pacemaker continued to pace the heart. However, hearts again reversed when fluid was removed.

C. *The effect of altering the tension (blood pressure) in the heart wall*

Exposed hearts were recorded from with suction electrodes up to 12 hours with little change in heart activity. When the stroke volume was reduced to about half by controlled bleeding, hearts reversed, became irregular for a few minutes and decreased in frequency (average 20%). In some hearts no reversals occurred during eight hours of recording. Body wall stimulation which occluded blood vessels increased diastolic filling and decreased the reversal-period and increased the beat frequency. When these animals relaxed again, stroke volume and beat frequency decreased and the duration of the reversal-periods increased (see v. Skramlik, 1930b). Reversal-pauses occurred in all of the above experimental conditions except when either hearts were overdistended with blood or collapsed in which case reversals were by pacemaker competition every 4–8 minutes.

Pacemaker competition reversals were produced when either the artery was temporarily occluded or when the outflow valve in an artificial circulatory system (Fig. 3) was temporarily closed.

Hearts which were ligated while *in situ* in an overdistended condition showed either irregular activity or the branchial pacemaker remained dominant. In this condition, the C pacemaker (v. Skramlik, 1926a) located in the middle of the heart sometimes paced the heart and alternated with the branchial pacemaker (*cf.* Mislin, 1964). Regular reversals and frequencies of contraction were established by simply decreasing the amount of blood. The amount of intracardiac blood was reduced to one-half the normal volume in eleven isolated cannulated hearts. This procedure usually resulted in a reversal followed by a short period of irregular activity. The average duration of the pulsation-series was reduced from 15 to 4 minutes, the frequency of contraction dropped 18% (Fig. 9), and the mode of reversal changed from those with reversal-pauses to pacemaker competitions. In hearts containing about one-half the normal volume of blood, contractions sometimes alternated in direction or alternated every few beats.

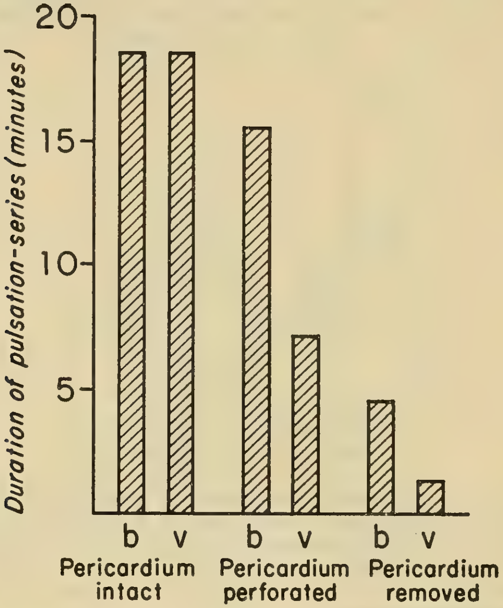

FIGURE 8. The decrease in the duration of the pulsation-series in isolated hearts due to a decrease in pericardial pressure and subsequent removal of the pericardium. Abscissa: (b) Branchial pacemaker frequency; (v) Visceral pacemaker frequency. The first two bars represent the controls with the pericardium intact. A perforation was first placed in the pericardium to eliminate the pericardial pressure. Then one surface of the pericardium was removed to expose the heart. The bars represent the average duration of the pulsation-series of 6 hearts.

It was observed that each alternating wave of contraction pumped the blood to the opposite arm of the heart. When hearts were completely collapsed, reversals were at first irregular and beat frequency decreased by 28% (Fig. 9).

The optimal intracardiac pressure for the greatest steady-state frequency in cannulated hearts was found to be 5 mm. water (Fig. 10). The initial frequencies after an increase in applied pressure were up to five times the steady-state frequencies and showed the characteristics of adaptation. When the intracardiac pressure was increased so that peristalsis could not occur, the branchial pacemaker remained dominant. Reversals reappeared when the intracardiac pressure was reduced. Reversals were induced in 4 out of 6 preparations by increasing intracardiac blood pressure from 0 to 2 mm. water in the nonpacing arm of the heart.

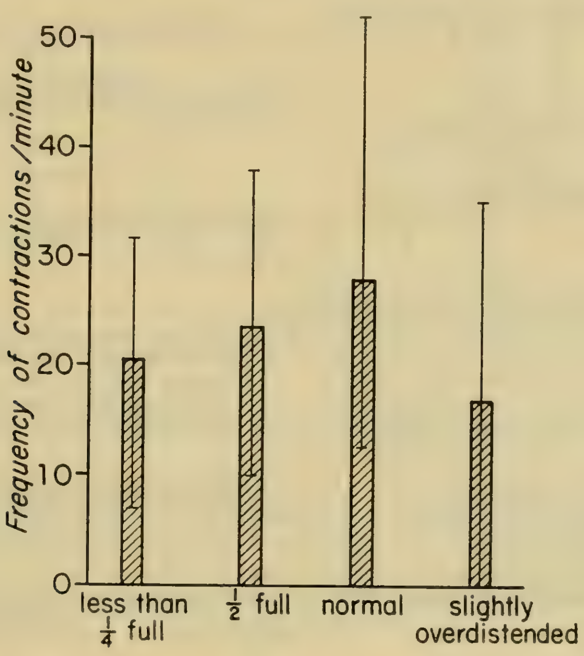

FIGURE 9. The effect of intracardiac blood volume on the frequency of a pacemaker. Abscissa: Less than ¼, ½, normal and slightly overdistended represent the degree of inflation of the heart by sea water or blood. Normal corresponds to the amount of blood which just inflates the heart. The bar is an average of 22 pacemaker frequencies (11 branchial and 11 visceral). The range is represented by the center line within the bar. These are steady-state frequencies.

D. *Pacemaker activity in half-heart preparations*

Twenty-five isolated *Ciona* hearts were cut in half after the heart activity was recorded for one hour and the inactive pacemaker began within 1–30 sec. (median, 6 seconds) even if a reversal had just occurred. Forty of the half-heart preparations contracted irregularly and 10 continued rhythmically for up to 8 hours.

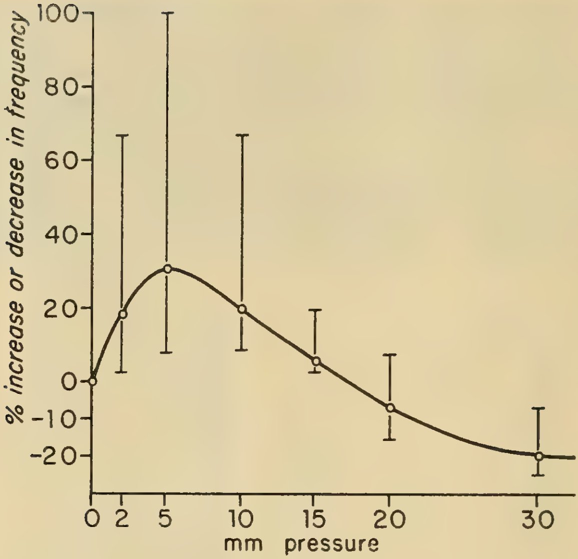

FIGURE 10. The effect of intracardiac pressure on the pacemaker frequency in isolated hearts (pericardium removed). Ordinate: % increase or decrease in frequency when the intracardiac pressure was raised from 0 to 2, 5, 10, 20, and 30 mm. water pressure. Abscissa: mm. intracardiac water pressure. The circles represent the average frequency change in 6 pacemakers. The ranges are indicated by the vertical lines. These are steady-state frequencies. The initial changes in frequency were greater.

Most half-heart preparations which were initially irregular became regular within 10 minutes and a few showed regular periods of activity and rest (Fig. 11) which did not correspond to the duration of the pulsation-series before sectioning the heart. Slowing of the pacemaker before stopping, as occurs in hearts *in situ* did not always occur. Periodic activity in half-hearts was changed or abolished in the same manner as hearts *in situ* by altering the intracardiac blood volume (pressure).

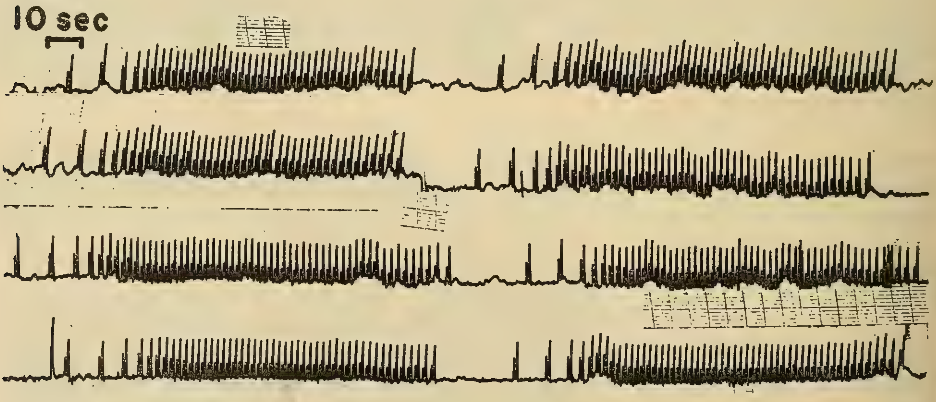

FIGURE 11. Continuous mechanical record from a half-heart showing periods of activity.

E. *Electrical stimulation of the heart*

Ipsilateral extrasystoles produced by electrical stimulation were followed by a compensatory pause. Electrical stimulation demonstrated a relative and absolute refractory period. It was possible to reverse both *in situ* and isolated hearts by stimulating the non-pacing arm of the heart. A pacemaker competition occurred when the post-stimulatory frequency of the newly active pacemaker was only slightly greater than the frequency of the previously active pacemaker. When the post-stimulatory frequency of the newly active pacemaker was lower, an abortive pacemaker competition ensued. These results show that an active pacemaker may not be driven (*cf.* Fig. 12).

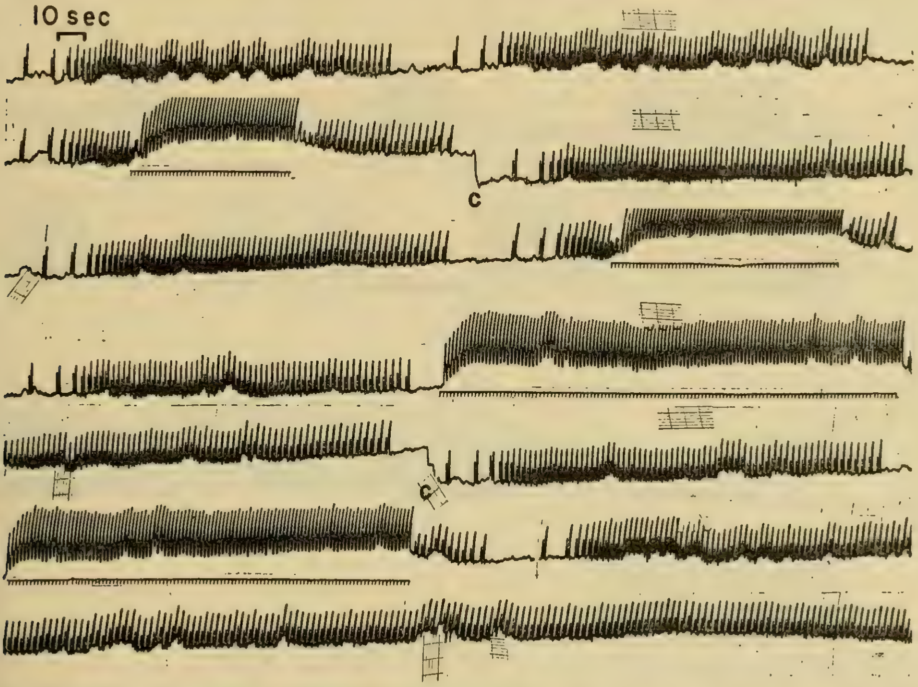

FIGURE 12. The effect of electrically driving an isolated heart which had only one active pacemaker. This continuous record is a continuation of Figure 11. The intact pacemaker showed periods of activity which were not changed by stimulating the heart from the contralateral end. Note that the quiescent periods fall in the middle and ends of the figure except in the last trace. The intracardiac blood volume was increased during the last trace, abolishing quiescent periods. The ink writer pen was changed at (c). Stimulation is indicated by the hash marks.

To test the oscillation reversal theory (see Discussion), the ends of hearts were stimulated with stimulators set at slightly different frequencies. The durations of the reversal-periods and competition reversals were determined by the frequencies and the difference in the frequencies of the two stimulators (such as 12.0 and 12.2/min., 29.3 and 27.3/3min., or 10.0 and 10.1/min.)

F. *The effect of heating or cooling the pacemaker*

The Q_{10} of the frequency of contraction was found to be 2.0–2.3 (tempt. range 5–25° C.). It was possible to induce activity in a pacemaker which had stopped by locally increasing the temperature of the pacemaker region by a few degrees (Fig. 13). An active pacemaker was stopped by lowering the temperature (Fig. 13, v. Skramlik, 1926b).

A constant temperature differential of 2°, 5°, or 10° C. was maintained at the two ends of 10 hearts (5 *in situ* and 5 isolated). In two *in situ* and in three isolated hearts, the pacemaker maintained at the greater temperature continuously paced the heart up to 8 hours. In other hearts, the heated pacemaker either stopped or missed beats after 30 minutes to four hours at which time the formerly quiescent pacemaker became active, but the duration of activity was short since the heated pacemaker always started again so that a short competition reversal ensued.

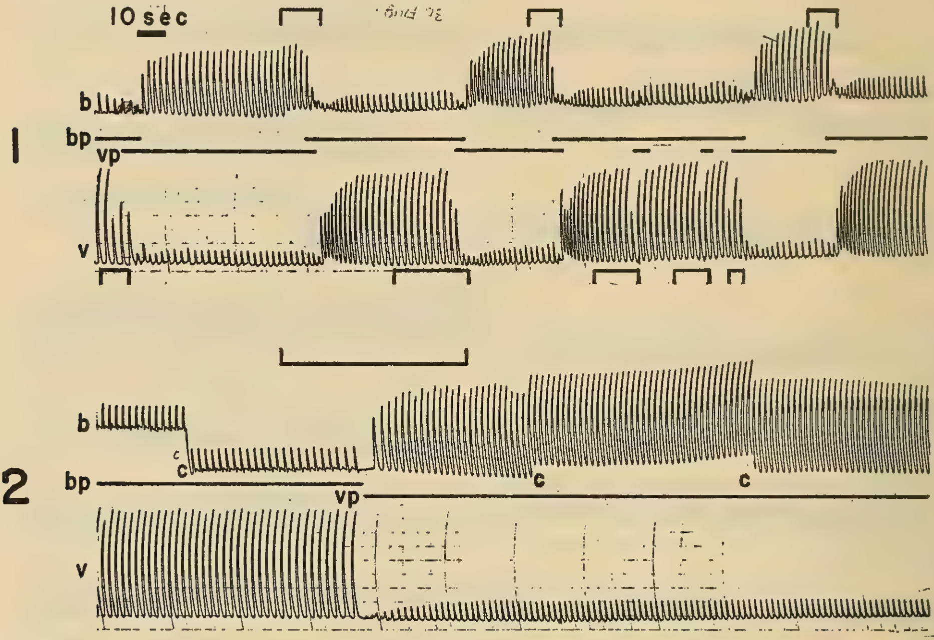

FIGURE 13. Reversals induced by locally heating the inactive pacemaker or cooling the active pacemaker. The bath temperature was maintained at 15° C. Localized warming (downward brackets) or cooling (upward brackets) was accomplished by running hot or cold water through a thermode positioned close to the pacemaker regions. Position of ink writer pen was changed at (c). Record 1. Reversals due to raising the temperature of the inactive pacemaker region by about 5° C. Record 2. Reversal due to cooling of the active pacemaker by 5° C.

In *Ascidia,* small *Chelyosoma* and *Corella* hearts, the reversal-periods were usually under three minutes but pacemakers could be kept active for over an hour by local heating.

G. *Cardiac output*

Cinematography was carried out at 24 frames/sec. on 6 *Ciona* hearts *in situ*. The start or end of a wave of contraction was determined to 2 frames (82 msec.). The velocity of peristalsis was 6.0 mm./sec. at 10° C.

Stroke volume was determined in four (30 mm. long, 2 mm. dia.) hearts which were contracting at about 20 beats/min. and reversing about every 20 minutes. The peristaltic wave of contraction completely occluded the lumen of the heart giving a stroke volume of 45 mm^3. Each pulsation-series circulated 13.5 cc. of blood which was equal to about the weight of the animal (tunic included). The cardiac output at 20 beats/min. was 0.9 cc./min. (6.6 cc. blood/100 g. body weight/min.).

During advisceral contractions, 2 mm. negative water pressure was applied to the visceral arm so that blood flowed into the cannula. Blood volume as determined by exsanguination was about 4% of the body (tunic included) weight. Thus, the blood was circulated about once every minute.

Blood flowed from the heart into the former venous system during the reversal-pause and during the diastolic periods of the first 3 to 5 contractions of pulsation-series in the 4 genera studied. During the first contractions of a pulsation-series, the stroke volume was up to twice as great as that after the first 5–10 contractions. It is evident that the arterial pressure increased over the venous pressure because (1) the peristaltic wave failed to form or only partially formed in the arterial arm of the heart after the first few contractions in a pulsation-series, (2) towards the end of a pulsation-series, part of the heart wall was forced into the vein (termed a contraction-valve), and (3) back-flow in the arteries became pronounced towards the end of a pulsation-series.

Discussion

A. *Performance of the heart*

It is apparent from the reviews of Seeliger (1893), Schultze (1901), v. Skramlik (1938) and Krijgsman (1956) that the reported frequencies of contraction, the durations of pulsation-series and modes of reversal in a given species greatly vary. Since it was demonstrated here that isolated hearts and hearts *in situ* can regularly reverse for 8 hours, many reported differences may have resulted from animals in poor condition.

Frequency of contraction

The heart rate depends upon the species, size and temperature of the animal. It was found here that the hearts of the smallest *Ascidia, Chelyosoma, Corella* and *Ciona* had the highest frequencies and the adults of the larger species (*Ciona, Chelyosoma*) had the lowest frequencies; and the Q_{10} of beat frequency was 2–2.3 in *Ciona*.

The frequency of the branchial pacemaker in *Ciona* hearts *in situ* has been reported by v. Skramlik (1938) and Bacq (1934a, 1935) to be about 10% greater than that of the visceral. This compares with the start of pulsation-series in isolated hearts (Fig. 6) and hearts *in situ* (see Results). Wolf (1932) and Jones (in Krijgsman, 1956) observed the frequency of both pacemakers to be the same, which was found in isolated and hearts *in situ* at the end of the pulsation-series (Fig. 6). Accleration at the start of a pulsation-series with reversal-pauses and then subsequent slowing before arrest has been observed in almost all species. Changes in frequency during pacemaker competitions usually did not occur.

Duration of the pulsation-series

In agreement with Roule (1884) and v. Skramlik (1926a, 1938) for *Ciona,* Schultze (1901) for salps and Burghause (1914) for *Pyrosoma,* it was found here that the pulsation-series were longer during the first hour or two after starting an experiment. In small *Ciona* advisceral pulsation-series were 2 to 4 times longer than abvisceral pulsation-series and the branchial pacemaker was almost always dominant in large animals. However, after the animals relaxed the durations of reversal-periods became nearly equal (every few minutes in small animals, 10–20 min. in larger animals).

Duration of the reversal-pause

The duration of the reversal-pause was found to be related to the size of the heart (and frequency because the longest hearts have the lowest frequency). The reversal-pause in large *Ciona* hearts was more than 5 seconds as compared to 2 to 3 seconds in smaller animals (*cf.* v. Skramlik, 1926a, 1938). When the length of the heart and the slow speed of peristalsis (1 to 2 cm./sec.) are considered, the apparent difference in the duration of the reversal-pause between large and small animals is reduced. Long reversal-pauses (over 10 sec.) resulting from overdistended or collapsed conditions do not reflect normal heart performance.

B. *Circulation of blood*

Rubber latex injections into the blood vessels of *Ciona* showed that the circulatory system is closed (Seeliger, 1893; Millar, 1953a). V. Skramlik (1929) demonstrated circulation in *Ciona* by injecting ink into the arterial blood vessel and observing its return in the venous end within one minute. Injection experiments have not been performed on other species, but Ritter (1893) on *Perophora annectens,* Schultze (1901) and Nicolai (1908) on salps, Burghause (1914) on *Pyrosoma giganteum,* Hecht (1918) on *Ascidia atra,* Herdman (1924) on *Botryllus,* and Koehnlein (1933) on *Phallusia* observed that blood flow reversed in the blood vessels whenever the heart reversed. It was found here that the amount of blood ejected during a pulsation-series was equal to the weight of the animal.

Diastolic filling

After puncturing the pericardium, hearts swelled with blood to occupy the entire pericardial space and either stopped or became irregular (*cf.* v. Skramlik, 1926a, 1938). However, rhythmical contractions resumed when intracardiac pressure was reduced, allowing the hearts to return to normal size.

When the blood vessels to the heart were severed and kept patent, blood in the heart was expelled until the isolated heart containd only about half the diastolic blood volume. Thus the pericardium is normally stretched by the intracardiac blood pressure which is transmitted to the pericardial fluid and the heart wall is relatively distensible compared to the pericardium.

A heart could not exert a suction to aid diastolic filling because the heart would have to expand against the pericardial pressure created by a relatively nondistensible pericardium, and there are no elastic elements in or on the 10 μ thick heart or pericardium having the geometry to create a negative pressure. Thus intracardiac blood pressure equals pericardial pressure and filling must result from positive venous pressure (*cf.* Krijgsman, 1956) and not by a suction force developed by the heart as proposed by Schultze for salps (1901), Burghause for *Pyrosoma* (1914), and v. Skramlik for *Ciona* (1938).

Formation of the peristaltic wave

The heart is composed of a single layer of musculoendothelial cells (Kawaguti and Ikemoto, 1958; Schulze, 1964) and conduction is by local current flow from muscle cell to muscle cell (Kriebel, 1967a and b).

The wave of contraction moved about 6 mm./sec. (Q_{10} 2.3, Carlson, 1906b) and was 2–3 mm., appearing as a twist which completely occluded the lumen, oriented 40–70° to the heart axis (10° C.). The velocity of the peristaltic wave was always the same in both arms of the *Ciona* heart although the visceral arm was usually longer (*cf.* Schultze, 1901; Nicolai, 1908; Hecht, 1918 and Waterman, 1943). Overdistention could slow the peristaltic wave; however, the velocity of the electrical wave of conduction is the same in each arm under all conditions of stretch. It is interesting to note that the Q_{10} of conduction velocity was the same as beat frequency (2.3; Kriebel, 1967a and b).

At normal temperatures, the beat frequency of *Ciona, Ascidia* and *Chelyosoma* hearts was high enough so that a new peristaltic wave began as soon as the previous one reached the arterial end, or shortly thereafter, which maintained a continuous flow of blood.

Formation of the functional valves in *Ciona*

The contraction valve (German, *Kontraktionszipfel*) is formed after the peristaltic wave of contraction passes over the first 1 to 3 mm. of heart wall and prevents backflow into the vein. During this period, the relaxed heart wall is forced into the venous ostium indicating that the pericardial pressure is greater than the venous pressure. As the peristaltic wave moves along the heart, the venous pressure increases over the pericardial pressure so the contraction valve disappears and blood flows into the heart. During the reversal-pause, back-flow occurs and the contraction valve re-forms. This observation demonstrates a pressure difference between the arterial and venous ends of the heart (*cf.* v. Skramlik, 1938). During a reversal-pause, back-flow is retarded by rest-folds (German, *Ruhefalte*) formed by a passive twisting of the heart wall (see Schultze, 1901; Heine, 1903; and v. Skramlik, 1930a). The following observations demonstrate that these valves are passively formed: (1) only one muscle cell type was observed in living hearts of *Ciona, Ascidia, Chelyosoma* and *Corella* under phase contrast; (2) *Ciona* and *Chelyosoma* myocardial cells have been recorded from intracellularly and no differences in action potentials were observed indicating cells with different functions (Kriebel, 1967a and b); (3) the rest-fold was observed to be formed progressively lower down the arterial arm during a pulsation-series, demonstrating that any portion of the wall can form the rest-fold. Also, the contraction valve formed only after the first few contractions of a pulsation-series concurrently with a fall in venous pressure (venous return) and during the reversal-pause; (4) the rest-folds and contraction valves disappeared after a slight increase in blood pressure; and (5) when hearts were opened into flat sheets electrical wave fronts moved uniformly along the heart (Kriebel, 1967a).

C. *Possible mechanisms of the beat reversal*

Extrinsic cardiac control

Hunter (1903b) proposed that reversal was due to nervous regulation because reversal-periods became irregular when *Molgula* hearts were isolated. This theory has received little support since completely isolated hearts of *Phallusia* (Lahille, 1890), *Ciona* (Loeb, 1900; Schultze, 1901; Carlson, 1906a; v. Skramlik, 1926a; Benazzi, 1935; Millar, 1952; Kriebel, 1963), *Ascidia atra* (Hecht, 1918) and *Corella, Ascidia callosa,* and *Chelyosoma* (this report) may exhibit reversal-pauses. Schultze (1901) has shown on salps that the pulsation-series are only slightly longer and the frequency of contraction slightly lower in isolated hearts. In addition, hearts *in situ* continue to beat in the absence of the ganglion (Day, 1921; Bacq, 1934b). However, there may be extrinsic regulation of the beat frequency (Carlson, 1906a; Hunter, 1903b; Burghause, 1914; Florey, 1951 and Kriebel, 1968).

Regulation of heart reversals by intrinsic ganglia

Since Alexandrowicz (1913) and Hunter (1903b) reported nerve cells in the heart, Burghause (1914) concluded that the heart activity was controlled by intrinsic nerve cells. However, subsequent investigators have not confirmed the presence of nerve cells (Millar, 1953a).

Ebara (1951, quoted in Krijgsman, 1956) noted that crushing the raphe stopped conduction, indicating that a through conduction system may be present connecting the two pacemaker regions. However, it has been demonstrated that crushing the raphe in *Ciona* hearts *in situ* does not alter the reversal-period or change the reversal-pause (Kriebel, 1967a and b). In addition, sectioning or removing the raphe and the undifferentiated line of cells in isolated hearts does not alter the conduction velocity or prevent reversal (Kriebel, 1967a and b).

Oscillation theory

Loeb (1900) proposed that reversal results from the pacemakers alternately "getting the upper hand." The two pacemakers can be compared to two oscillators set at slightly different frequencies (see Results). The pulses of the non-pacing oscillator would shift out of phase with respect to the refractory periods (*cf.* Schultze, 1901). The greater the difference in frequency of the two pacemakers, the shorter each pulsation-series becomes until only contralateral extrasystoles occur. An oscillatory system explains pacemaker competition reversals but does not account for the acceleration at the start of a pulsation-series and for the slowing before a reversal-pause.

Periodic pacemaker fatigue (Krijgsman, 1956)

The main supportive evidence for pacemaker fatigue is that isolated hearts may regularly reverse and half-heart preparations may show periods of activity and rest. However, the results presented here demonstrate that a change in the amount of blood within isolated hearts abolished or altered the mode of reversal. Isolated hearts with pericardium removed swelled within an hour, whereas swelling required several hours in hearts with an intact pericardium. These observations may explain why Millar (1952) found that newly isolated *Ciona* hearts beat in one direction for the first few hours before reversals occurred and why Haywood and Moon (1950, 1953) did not observe reversals in isolated *Ascidiella* and *Ciona* hearts for 10 hours.

Collapsed and overdistended hearts may beat in a given direction without slowing for 8-hour periods (see Results) indicating that pacemakers do not simply fatigue after a given number of beats. A pacemaker may slow and then accelerate during the time the heart should have been paced by the opposite pacemaker (*cf.* Lahille, 1890). Moreover the frequency of a pacemaker can be increased by increasing the intracardiac blood volume just before a reversal (see Results).

As found by v. Skramlik (1926a) on *Ciona* and Koehnlein (1933) on *Phallusia* it was noted here that a pacemaker continued to pace the heart up to 3 times the normal frequency as long as localized heat was applied. In addition, a pacemaker which had just stopped, started again when it was locally heated, the arterial vessel was occluded, the dominant pacemaker was locally cooled, or the heart was cut in two.

An isolated half-heart may show periods of activity and rest as demonstrated by Schultze (1901) and Ebara (1954) in *Salpa* hearts and by v. Skramlik (1926a), Millar (1952, 1953a, 1953b) and Jones (in Krijgsman, 1956) in *Ciona* and in this paper in *Ciona, Corella, Ascidia* and *Chelyosoma.* However, Lingle (in Loeb, 1900) on *Ciona,* Nicolai (1908) on salps and Hunter (1903a) on *Molgula* found that half-heart preparations continued to beat for long periods of time. In this investigation, some isolated half-hearts showed periods of activity and rest, but the periodicity in most cases was irregular, and generally did not correspond to periods of activity of the heart *in vivo* before isolation of the half-hearts. In all cases, the periodicity was either changed or abolished by altering the amount of fluid within the half-heart. It is very important to note that after 20 *Ciona* hearts were cut in two, 10 half-heart preparations continued to beat.

Back-pressure theory

Kuhl and v. Hasselt (1822), Ritter (1893) on *Perophora,* Schultze (1901) on salps, Heine (1903) on *Ciona* and *Salpa,* Hunter (1903b) on *Molgula,* v. Skramlik (1938) on *Ciona* and *Clavelina,* and this author on *Ciona, Corella, Chelyosoma* and

Ascidia observed back-flow during a reversal-pause, demonstrating that a "back-pressure" builds up during a pulsation-series.

Cinematographic analyses of beating *in situ* hearts showed that the stroke-volume gradually decreased during the first beats of a pulsation-series indicating a fall in venous pressure. During the reversal-pause and during diastole at the start of a pulsation-series, blood flowed through the heart towards the arterial end of the heart. Towards the end of a pulsation-series back-flow of blood increased so that blood sometimes oscillated in small vessels (see Results: *cf.* Pizon, 1900; v. Skramlik, 1938).

In addition to results shown here earlier workers demonstrated that the excitability of the pacemakers could be modified by stretch (blood pressure): (1) mechanical stimuli causing the animal to contract shortened the pulsation-series (Lahille, 1890; Bancroft and Esterly, 1903; Nicolai, 1908; Burghause, 1914; Koehnlein, 1933; v. Skramlik, 1930b and 1938; and Bacq, 1935) or lengthened the period (Schultze, 1901; Day, 1921); (2) Lahille (1890), Schultze (1901), Day (1921) and v. Skramlik (1926a, 1938) observed that a loss of blood in an intact animal changed the pulsation-period; (3) reversal was induced by occluding the artery (Lahille, 1890); (4) Koehnlein (1933) noted that poorly distended *Phallusia* hearts did not beat; and (5) an increase in bath temperature (Knoll, 1893; Koehnlein, 1933) or the addition of nicotine (Schultze, 1901) increased the frequency of contraction and reversal which can be attributed to a rapid build-up of arterial pressure since a local increase in temperature increases both the frequency and duration of a pulsation-series (see Results).

Many investigators have observed a gradual slowing towards the end of a pulsation-series, indicating a decrease in pacemaker excitability (Lahille, 1890, and Koehnlein, 1933, on *Phallusia;* Schultze, 1901, and Nicolai, 1908, on *Salpa;* Hunter, 1903b, on *Molgula;* Carlson, 1906a, v. Skramlik, 1926a, Wolf, 1932, Bacq, 1935, Benazzi, 1935, on *Ciona;* Carlson, 1906a, on *Clavellina;* Burghause, 1914, on *Pyrosoma;* Hecht, 1918, and Day, 1921, on *Ascidia;* Herdman, 1924, on *Botryllus;* and here on *Ciona, Ascidia, Corella* and *Chelyosoma*). The cannulation experiments reported here demonstrate that the pacemaker is extremely sensitive to changes in applied pressure and that the pacemaker shows the characteristics of adaptation.

These observations fulfill the first aspect of a reversal mechanism as posed by Haywood and Moon (1950, 1953; *cf.* v. Brücke, 1925); that is, a decrease in venous pressure decreases the excitability of the active pacemaker and adaptation of the pacemaker leads to arrest. Since the frequencies of pacemakers towards the end of a pulsation-series in both directions of contraction were found to be the same (Fig. 6) and since the excitability of a pacemaker is related to the blood pressure, the second aspect of a back-pressure theory as posed by Haywood and Moon is satisfied; that is, the excitability of the former arterial pacemaker would be greater than that of the venous pacemaker due to the greater blood pressure.

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SUMMARY

1. Experiments were conducted on isolated and *in situ* hearts of 4 species of tunicates in order to investigate beat reversal. The mechanical and electrical properties of *in situ* and isolated hearts were found to be the same. The intracardiac pressure was found to affect the excitability of pacemakers and it could be adjusted so that reversals did not occur, indicating that pacemakers did not fatigue. Small changes in the intracardiac pressure (or intracardiac blood volume) altered beat frequency and the duration of a pulsation-series. The greatest beat frequency was obtained with 5-mm. intracardiac water pressure. After a change in intracardiac pressure, pacemakers showed the characteristics of adaptation.

2. Localized heating of an active pacemaker or localized cooling of an active pacemaker initiated reversal. Pacemaker regions under localized heating remained active for periods of 8 hours without slowing.

3. Pacemakers which had just stopped after a pulsation-series became active again when either the hearts were bisected, the pacemaker region was locally heated, the surrounding myocardium was stimulated electrically, the arterial vessel was occluded or the venous pressure was increased.

4. Two modes of beat reversal were observed: (1) hearts in relaxed animals decreased in frequency and stopped, after a few seconds the formerly inactive pacemaker increased in frequency, reached a steady frequency, slowed and then stopped; the cycle was repeated; (2) pacemakers in most isolated hearts and in hearts in damaged or contracted animals alternated in dominance after periods in which they were both active. It is suggested that reversals occurring after pauses in pacemaker activity result from a decrease in venous pressure which lowers pacemaker excitability and adaptation of the pacemaker which leads to its arrest; and an increase in arterial pressure which raises the excitability of the previously inactive pacemaker so it begins after the reversal-pause. The second mode of reversal results from a shift in phase of the refractory period of the heart (at each end) and the continuous activity of both pacemakers. Similar reversals were produced when the ends of the heart were electrically stimulated with two stimulators set at different frequencies.

5. Cinematographic analyses of hearts *in vivo* demonstrate that the average velocity of the wave of contraction was the same in both directions of contraction (6 mm./sec. at 10° C.) and that there was a gradual decrease in diastolic filling and cardiac output during a pulsation-series.

6. The stroke volume in 8 cm. long *Ciona* (heart 30 mm. long) was 45 mm^3. At 20 beats/min., this was 6.6 cc. blood/min./100 g. animal. The blood volume was determined to be 4% of the body weight by exsanguination. During a *Ciona* heart pulsation-series, 13.5 cc. of blood were ejected, which was equal to the weight of the animal, demonstrating that blood returns to the heart and does not oscillate.

7. The formation of the functional valves in the heart wall (contraction valve and rest-fold) was concluded to be due to differences in arterial and venous blood pressure since there are no specialized areas of the heart wall.

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THE FEEDING AND SWIMMING OF CONCHOECIA (CRUSTACEA, OSTRACODA) [1]

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At the Naples Zoological Station, in the spring of 1935, I studied the feeding and swimming of living *Conchoecia.* Judging from the description by Müller (1894), my specimens were probably nearly all of the species *C. spinirostris* Claus. A few specimens of a different species, probably *C. curta* Lubbock (= *C. clausii* Sars), were recorded as feeding like the others.

Careful notes of my observations were made, and many specimens were preserved for an intended anatomical study of the appendages. Subsequent loss of the specimens prevented this study, but Iles (1961) has now given a very thorough description of the appendages of *Conchoecia borealis antipoda.* Iles worked only with preserved specimens, yet includes a discussion of probable swimming and feeding mechanisms, as inferred from the anatomy and from the brief comments of Müller (1894) on living animals.

Tranter (1962), in a review of Iles' work, regrets the lack of direct observations on living material. Thus, a report of my observations would now seem to be appropriate.

MATERIAL AND METHODS

During favorable weather, fresh plankton from a depth of about 130 meters in the Bay of Naples was received daily. Numerous specimens of *Conchoecia,* together with other crustaceans, were transferred from the plankton samples to large jars of clean sea water, in which *Conchoecia* usually lived several days, but showed maximum activity only during the first hour.

Observations were made of living specimens swimming in the jars and in embryological watch glasses viewed under a stereoscopic microscope. Further details of the methods used are given in the following section.

OBSERVATIONS

Locomotion

Freshly caught *Conchoecia* swam almost continuously, at about the speed of the fastest copepods. Swimming was in a zigzag series of darts, as in the copepods, except that the overall course was more irregular, with each dart usually a rolling curve rather than a straight line. The power for swimming was seen to be provided entirely by the very rapid beating of the antennal exopods. It seemed that during swimming there was usually a moderate gape between the two halves of the carapace, but observations on this point were not extensive.

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[1] This research was aided by the appointment of the author to the Cambridge University Table at the Naples Zoological Station.

Individuals not beating the antennae sank rather rapidly to the bottom. After about the first hour, all individuals spent much time resting, but out of some thirty specimens in a jar there were always about five or six darting about in the manner described above. The resting specimens often appeared to run short distances along the bottom, doing so, however, not by the use of any leglike appendages, but by a rapid beating of the antennal exopods at an amplitude too slight to cause swimming. When kicking movements were made by the mandibular palps, maxillae, and first trunk limbs, their action pushed the animal if anything more backwards than forward.

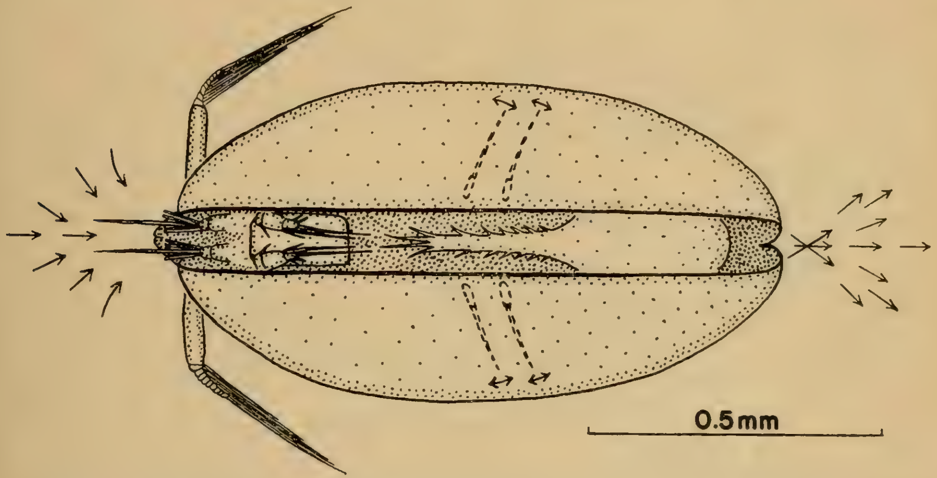

FIGURE 1. Ventral view of a living *Conchoecia spinirostris*, from a rough, freehand sketch, modified to conform partially with published camera lucida drawings. Some appendages omitted in whole or in part. Double-headed arrows indicate amplitude of beat of the vibratory plates. Other arrows show currents seen with the aid of carmine particles.

When an individual found food and started feeding, it always immediately ceased swimming, so that it sank to the bottom if not already there.

Feeding

The specimens of *Conchoecia* observed at Naples fed almost exclusively on dead crustaceans (mostly copepods, a few *Conchoecia*, and some larval malacostracans). Active individuals of *Conchoecia* jumped onto such food and then held it against the ventral gape of the carapace. This gape remained moderate throughout feeding (Fig. 1), a wider gape being found only in moribund individuals. The food was held and constantly shifted in position chiefly by the mandibular palps, aided by the exopods of the maxillae and first trunk limbs. If the food was smaller than the ostracod, it was usually rotated in the manner indicated in Figure 2. Often, when the food was being manipulated, the hold upon it was momentarily released, its loss being prevented by the copious, very sticky secretion of the marginal carapace glands. Sometimes the caudal furca lifted food partially away, permitting the latter then to be pushed by the appendages into a new position.

The continual shifting of the food served to press different parts of it successively against the gnathobases of the mandibles. Each mandible was in constant motion, rotating first one way and then the other about an articulation near the tip of the labrum. The rotation that adducted the gnathobases appeared to move the dorsal end of the coxa anteromedially and the tip of the palp in the opposite direction (Fig. 3). Independent and much more extensive movements of the palp were made when it was holding and manipulating the food.

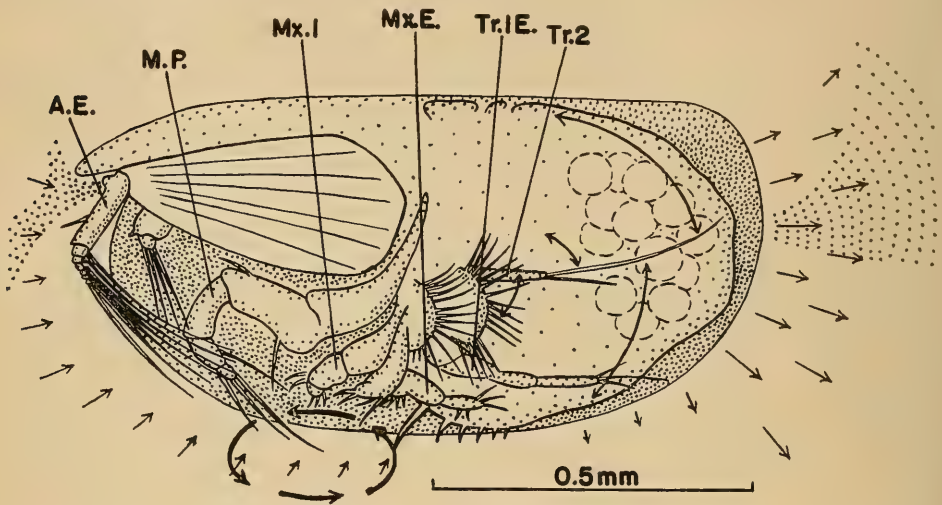

FIGURE 2. Side view of a living female *Conchoecia spinirostris,* drawn in same way as Figure 1. Double-headed arrows delineate sweeping movements of second trunk limb and of its long seta. Heavy arrows outline the rotary movement of large food. Thinner arrows show currents revealed by carmine particles. Stippling outside the animal shows current-patterns revealed by *Sepia* ink.

A.E., antennal exopod; M.P., mandibular palp; Mx.l., maxillule; Mx.E., exopod of maxilla; Tr. 1 E., exopod of 1st trunk limb; Tr. 2, 2nd trunk limb.

If the food was an intact crustacean, some minutes usually elapsed before its exoskeleton was punctured by the activities just described. Eventually, however, punctures would be effected in one or more places, and there would then follow a relatively rapid breaking up of the corpse into small pieces that were quickly swallowed. Harpacticoid copepods, which I had punctured with a needle, were completely eaten in about half a minute.

Although the breaking up of the food was accomplished chiefly by the mandibles, some aid seemed to be provided by the maxillules and maxillae. These appendages showed no rotary motion, but rather seemed alternately to push forward and inward and then retreat. In the case of the maxillae, their exopods showed independent movements for holding and positioning the food, as already described.

The esophagus of *Conchoecia* is rather narrow and it was not observed to dilate appreciably during swallowing. At frequent intervals food could be seen passing up the esophagus smoothly and quickly, in the form of small cylindrical boluses. Since entire crustaceans were eaten, these boluses must have included compressed fragments of exoskeleton.

Perhaps dead animals other than Crustacea are sometimes eaten, but no such instances were observed. On one occasion, *Conchoecia* was seen trying to feed on a dead fish larva (*Syngnathus* sp.), but the scales already present prevented success. Often, the guts of freshly caught *Conchoecia* were bright red, suggesting vertebrate food. But negative results with the benzidine test, applied to a number of specimens, indicated that hemoglobin was absent. Probably the red color came from a pigment of similar hue seen in parts of some of the copepods accompanying the *Conchoecia*.

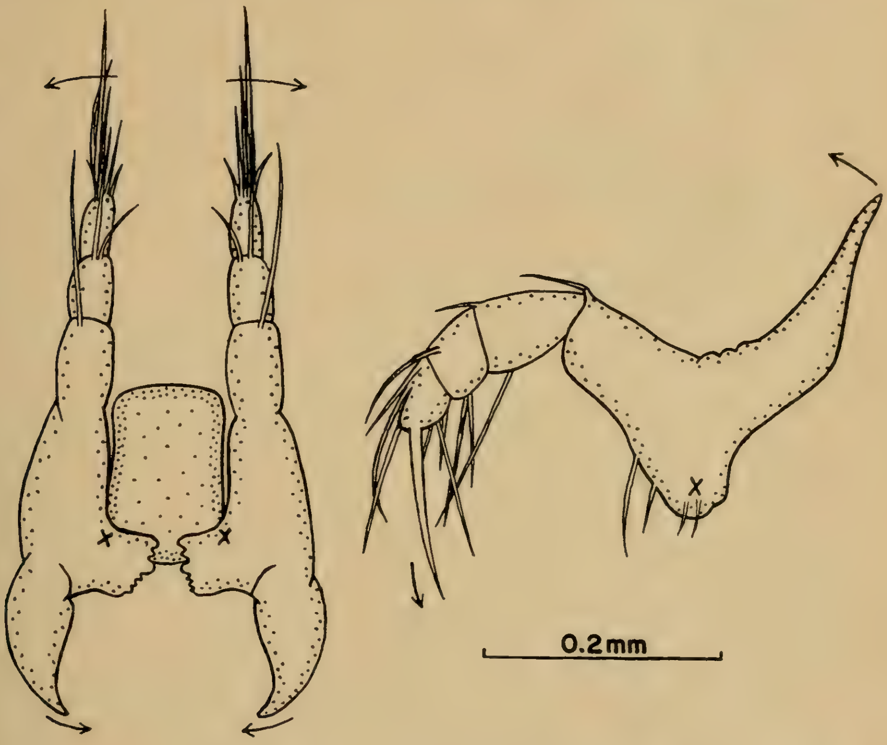

FIGURE 3. Ventral and side views of the mandible of *Conchoecia* drawn in same way as Figure 1. The arrows indicate the rotary motion seen in living specimens, the center of articulation being marked 'X.'

Sometimes, *Conchoecia* was observed eating compact masses of detritus, probably in most cases derived from fecal pellets.

Only once was *Conchoecia* seen to feed on a living crustacean. The prey on this occasion was a large euphausiid larva, being attacked by about six *Conchoecia* simultaneously. At first the larva was still able to flex its body, kick strongly with the abdomen, and maintain a rhythmic beat with some of the appendages. The ostracods swarmed all over it, sometimes momentarily knocking one another off, but never being dislodged by the struggles of the larva. After a good many minutes the first puncture through the exoskeleton was achieved, and thereafter feeding was rapid, soon resulting in the death of the prey.

It seems likely that the euphausiid larva was already partially moribund when first attacked by the ostracods. This conclusion is supported by the results of many experiments in which single specimens of *Conchoecia* were isolated in crystallizing dishes with known numbers of other living organisms, such as harpacticoids, calanoids, and annelid larvae. Counts after twenty-four hours only once revealed a single missing copepod, which may quite possibly have died or become moribund before being eaten.

Some simple attempts were made to discover how *Conchoecia* finds its food. Specimens were watched that were swimming in an embryological watch glass containing the body of a crustacean recently punctured with a needle. On one typical occasion, a specimen brushed repeatedly against a dead harpacticoid without attempting to feed. Yet, when this ostracod paused after twenty minutes to rest on its back and the harpacticoid was then laid along the ventral gape of the carapace, very active feeding began at once. On another occasion, a dead harpacticoid was not found and eaten by one of two ostracods until after forty minutes. But, when another dead harpacticoid was now added, the same ostracod at once became extremely active and almost instantly found and ate the food, the second ostracod, meanwhile, showing no noticeable change in activity.

Once having started feeding, specimens of *Conchoecia* showed great tenacity and were not easily disturbed. Feeding even continued when specimens were transferred by pipette from one dish to another, a fact which made many of the microscopic observations possible.

The question as to whether or not *Conchoecia* can feed on suspended particles was studied rather carefully. By watching carmine particles it was found that the constant beating of the vibratory plates on the maxillae and first trunk limbs produced a weak current, distributed as shown in Figures 1 and 2. Figure 2 also shows the pattern of this current, rather faintly revealed by the use of ink from a living *Sepia*. Any particles passing back between the valves of the carapace were observed with particular care. They could be seen moving back at all levels, up to where the carapace attaches to the body. Usually, a particle could be followed until it left in the posterior current. Never were any seen to be filtered out, except sometimes on the setae of the vibratory plates. When a dense suspension of particles was used, the setae of these plates became rather clogged. But there are no structures for combing these setae, and so they remained clogged even twenty-four hours after the animals had been returned to clear water.

In addition to the currents just discussed, local swirls were seen, caused especially by sweeping movements of the exopods of the maxillae and first trunk limbs, and to a lesser extent by similar movements of the mandibular palps. These swirls brought in particles which became trapped in the sticky secretion of the marginal carapace glands. If the particles were numerous, boluses soon formed, which the animal tried to remove by means of the caudal furca. Because of the sticky secretion, such removal was difficult. On the other hand, intermittent movements of the maxillae, maxillules, and mandibles tended to push the boluses toward the mouth.

Those that contained *Sepia* ink usually were swallowed, but ingestion of carmine was never seen.

The sticky secretion, while usually copious, was occasionally scanty or almost absent. A few attempts to determine its chemical nature gave only negative results; it was not selectively stained by methylene blue, toluidine blue, thionine, or Meyer's muci-carmine.

One pair of appendages, namely, the second trunk limbs, did not share in any of the activities discussed thus far. The long, very flexible seta on each of these limbs appeared to be used for cleaning the posterior parts of the body, sweeping in a wide arc as shown in Figure 2. Over the ventral half of this arc the limb moved as a whole, flexing at its joint with the body, but the arc was continued dorsally only by the distal seta, which flexed at a point just proximal to its base.

Discussion

In discussing the swimming of *Conchoecia*, Iles postulates a slight uplift of the anterior part of the body, and he also suggests that at times swimming may continue when the valves of the carapace are tightly shut. Neither of these modes of swimming was seen during my observations at Naples, although both conceivably might occur under some circumstances. The special mechanism for opening the valves of the carapace is described by Dennell (1960, p. 451).

Müller (1894) gave reasons for believing that *Conchoecia* lives mainly on the bottom. This view was refuted by Iles, whose arguments are supported by the observations reported in the present paper. The almost continual swimming seen in freshly caught specimens is probably typical for *Conchoecia* living in the sea. Specimens that ceased swimming, as when feeding, were seen to sink rapidly, and probably they would do so at all depths (Marshall, 1954; Sundnes, Leivestad, and Iversen, 1965). Although the cuticle of the carapace is much thinner in *Conchoecia* than in bottom-living ostracods (Harding, 1964), the content of hydrocarbons and lipids, that might provide buoyancy, has been reported to be unusually low (Blumer, Mullin, and Thomas, 1964). Active swimming would thus be necessary for staying at any particular depth, as well as for the vertical migrations that have been reported (Hure, 1955; Iles, 1953; McHardy and Bary, 1965; Motoda and Anraku, 1954, 1955; Vinogradov, 1962).

Figures reported for the carbohydrate content of *Conchoecia* (Raymont and Conover, 1961) suggest that total energy requirements could not be met from this source for more than about thirty-six hours (assuming complete utilization of the carbohydrate and a respiratory rate similar to that in *Calanus*). If these figures are typical, and if the lipid content is normally very low (Blumer, Mullin and Thomas, 1964), it would seem that *Conchoecia* must be able to find food at frequent intervals.

From his anatomical studies, Iles inferred that *Conchoecia* preys on living crustaceans. Only one possible instance of such predation was observed at Naples, and even then the prey may have been moribund when attacked. On all other occasions, only dead crustaceans were eaten, despite the presence of numerous living crustaceans from the ostracods' own habitat. Kane (1963) found that an oceanic amphipod began to attack living *Artemia* only after it had fed for several days on dead specimens, but that may have been because the prey was unfamiliar.

Conchoecia seemed to find its food merely by chance encounter, with sight and smell playing little or no part. However, one individual which had eaten a dead harpacticoid was very quick in seizing a second harpacticoid of the same kind. Thus, smell may be important after appropriate conditioning, and possibly living Crustacea were not attacked in the laboratory only because releasing stimuli were absent.

For *Conchoecia* when eating dead crustaceans, the feeding mechanism I observed was in most essentials like that postulated by Iles. Perhaps the chief feature not described by Iles was the constant shifting and rotation of the food, accomplished by the mandibular palps, the exopods of the maxillae and first trunk limbs, and sometimes even the caudal furca. During this manipulation, the appendages did not always have to grasp the food, thanks to the presence of the sticky secretion from the marginal carapace glands. Probably such retention of large food is the chief function of this secretion.

In the living animal, the mandible was seen to rotate about a point near the posterior end of the labrum, just as would be predicted from Iles' fine description of the anatomy. But the movement seen at the dorsal end of the mandibular coxa (Fig. 3) suggests a much looser articulation of the dorsal condyle than would be inferred from Iles' account. The socket for this condyle was not described by Iles, and perhaps it is soft or is in the form of a groove. On the other hand, possibly the movement seen was merely the turning of a flange, although no suitably oriented flange has been described.

Food was seen to be swallowed by *Conchoecia* in much smaller boluses than would be expected from the size of some of the objects found by Iles in the stomach. Presumably, such items as pieces of exoskeleton are much folded and compressed before being swallowed.

For the possible capture of particulate food by *Conchoecia,* Iles has suggested two mechanisms. One of these receives no support from my observations, there apparently being no significant retention of particles from the current produced by the beating of the vibratory plates. This current is probably only for respiration and for drawing in water across the chemoreceptors of the antennules (see Fig. 1). On the other hand, my work does provide some slight support for the other mechanism Iles has suggested. The sticky secretion of the marginal carapace glands was seen to trap many particles brought in by swirls created by the more leglike appendages. Further movements of the appendages then formed the trapped particles into boluses that were pushed toward the mouth. Carmine particles were not swallowed (as noted, indirectly, by Müller, 1894), but ingestion of *Sepia* ink did occur. However, this last result was seen only in relatively dense suspensions of particles, such as would rarely be encountered in the normal habitat. Furthermore, the mechanism that secured the boluses was merely that used for larger food, and in most cases the animal attempted to push each bolus away with its caudal furca. Thus, any significant capture of particulate food by *Conchoecia* appears doubtful. It is true that diatoms and detritus have been found in the stomach of *Conchoecia* (Iles, 1961; Vinogradov, 1962), but these items may have come from fecal pellets, which apparently were among the things eaten by the *Conchoecia* that I observed.

I owe the warmest thanks to the staff at the Naples Zoological Station, for providing every possible assistance, and to Professor H. Janetschek and his staff, for their generous hospitality at the Institute of Zoology, University of Innsbruck, where part of this paper was written.

SUMMARY

1. The swimming and feeding of living *Conchoecia* is described. In general, the observations reported support conclusions arrived at by Iles from anatomical studies.

2. Freshly caught animals swam constantly, except during feeding. Apparent running over the bottom was achieved by beating of the antennal exopods, as in swimming, and was not the result of any action by the more leglike appendages.

3. The food consisted almost entirely of dead crustaceans. Formed masses of detritus were also eaten. Living Crustacea were generally ignored, although one attack on a possibly moribund specimen was observed.

4. There was much shifting and rotation of the food by the mandibular palps, the exopods of the maxillae and first trunk limbs, and the caudal furca. This manipulation served to press different parts of the food against the mandibular gnathobases. Loss of food was prevented by the sticky secretion of the marginal carapace glands.

5. The dorsal condyle of the mandible seemed to be more loosely articulated than would appear from the description by Iles.

6. There was no significant filtration of particles from the current produced by the beating of the vibratory plates.

7. Swirls caused by the more leglike appendages brought in particles which became trapped in the sticky secretion of the marginal carapace glands. Resulting boluses of *Sepia* ink particles were pushed to the mouth and swallowed. However, there are reasons to doubt the significance of this mechanism in the natural habitat.

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REVERSAL OF CELL LAYERS IN HYDRA: A CRITICAL RE-APPRAISAL

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One of the earliest experiments performed with hydra was to evert single polyps and observe their behavior (Trembley, 1744). After the animal was everted by any of several similar techniques, it either died, returned to its original orientation or survived without any obvious re-turning. The last of these three responses to everting has given rise to a multiplicity of explanations (Nussbaum, 1887, 1891; Roudabush, 1933; Weissman, 1890; Ischikawa, 1890; Trembley, 1744). Weissman and Ischikawa maintained that everted hydra always regained their normal body layer orientation by reversion of the body layers through the oral or aboral pores. The reversion resembles the manner in which a glove is everted when being removed. In the case of everted animals pierced with bristles to prevent reversion, they predicted turning through the mouth or around "rifts and ruptures" (Weissman). Ischikawa (1890) drew many imaginary pictures to illustrate how an everted hydra pierced with a bristle might restore its normal cell layer orientation.

Nussbaum (1891) on the other hand attributes the return of the animal's two cell layers to their normal orientation to a rather unique phenomenon. He says that a new ectodermal layer and mesoglea are formed on the outside of the everted animal advancing from open or damaged areas along the body column. He describes histological sections which give the appearance to him of a multilayered structure consisting of ectoderm, mesoglea, endoderm, mesoglea, ectoderm. Not being able to distinguish the two endodermal layers, he mistakenly suggested that new tissue had formed whereas what he was observing was two opposed hydra epithelia. To account for the extra ectodermal layer left in the gut after the formation of a new ectoderm on the outside of the everted animal, Nussbaum suggests that these cells and extra mesoglea are resorbed.

Trembley (1744, quoted in Nussbaum, 1891), however, maintained that everted animals which had been pierced with bristles restored their normal body layer orientation by either cell migration or that the ectodermal cells became re-formed into endodermal cells and endodermal cells into ectodermal cells. Lacking histological techniques, Trembley was unable to check either of these hypotheses. However, Roudabush (1933) repeated the eversion experiments and concluded that in an everted animal, that is not seen to re-turn, the cell layers become reorientated by cell migration. To permit cell migration, Roudabush suggests that the mesoglea disappears to permit cell migration and then re-forms. The cells of the two layers are said to migrate in opposite directions so as to re-establish their normal orientation.

In this study the behavior of the two hydra body layers has been carefully studied in everted animals. In particular, the explanations given by Roudabush have been examined and found not to be in agreement with experimental observations.

Materials and Methods

All experiments were conducted with *H. pseudoligactis* grown under constant temperature of 70 ± 5° F. and constant light. The animals were fed *Artemia salina* nauplii every second day and maintained in a standard culture solution containing 0.02% w/v $CaCl_2 \cdot 2H_2O$, 0.01% w/v $NaHCO_3$, and 0.005% w/v Na_4EDTA in distilled water (Loomis and Lenhoff, 1956). Only animals starved 24 to 48 hours were used in experiments.

To prepare the everted animals, a hydra was lightly grasped below the tentacles with a pair of watchmaker's forceps. A second pair of sharpened forceps was inserted through the animal's mouth after it had been stimulated to contract. The basal disc was grasped with the second pair of forceps and pulled through the mouth. For some experiments with everted animals the gastrodermis was stained by placing three drops of 1% neutral red in 250 ml. of culture solution. In about 24 hours only the gastrodermal cells are deeply stained. After staining, the animals were everted.

Greater than 95% of all animals everted remained healthy during the experimental period. In later experiments as technique and skill improved, 100% survival was achieved.

Histological sections were prepared by fixing in Bouin's fixative for a minimum of three hours. After embedding, paraffin sections were cut at 5 microns and stained with toluidine blue buffered to pH 8.

Results and Discussion

The experiments with everted animals were originally planned so that the effect of the ionic compositions of the normal culture medium on the gastric cells could be observed (Macklin and Burnett, 1966). It was impossible to consistently maintain animals in the everted position without pinning them down and so this technique was abandoned in favor of the more easily controlled technique of pinning an opened gastric piece on cork. However, the study of the everted animals raised an important question relating to the mechanism of regeneration of the everted animals. Roudabush (1933) described two methods for regeneration of everted animals: (1) The body column is observed to be peeled back in the manner in which a sock may be turned inside out when it is being removed. (2) The mesoglea is broken down and cells migrate so that the body layers are interchanged and the normal arrangement is reestablished.

If Roudabush's description is correct, then in the examination of many animals, intermediate stages should be easy to find. He states that in 8 hours essentially all animals have resumed their normal form. This has been confirmed in this study. Yet no intermediate stages were found during this time period. Either an animal was everted or it was not when examined histologically. *Histological sections were only prepared from animals which were not observed turning back.*

Most animals had turned in 4 hours with a few remaining everted for longer periods. Figure 1 shows an animal 1.5 hours after being everted. Figure 2 is an animal that has remained everted for 24 hours. Note no cell migration can be seen.

No cell migration was observed in this study which included histological examination of everted animals at the following times after everting: 0.25, 1.5, 2.5, 3.5, 4, 4.5, 5, 5.5, 7.5, 10, 19, 24, 48, 72, 96, 120, and 144 hours. Animals were also everted and pinned to cork. These later animals also showed no evidence of cell migration and were all still everted after 5.5 hours. There is an obvious discrepancy between these observations and those reported by Roudabush.

All micrographs, unless noted, are 5-micron longitudinal sections stained with toluidine blue, pH 8. All everted animals were incubated in standard culture solution; and time was measured from time of everting to time of fixation. E = epidermis, GD = gastrodermis, ME = mesoglea, C = cnidoblast, G = gland cell. Bar in figures is 100-micron scale.

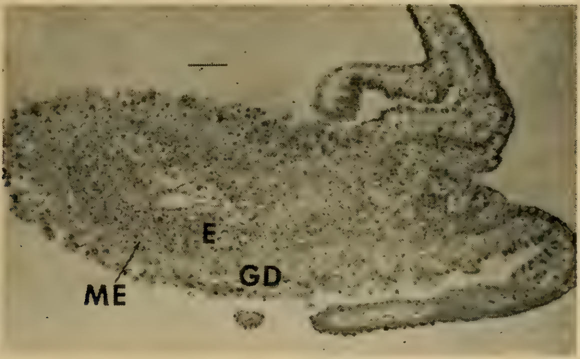

FIGURE 1. Section of animal everted for 1.5 hours. Note the persistence of the distinct cell layers.

Roudabush everted animals by pushing the basal disc through the mouth with a glass needle and then everting the animal onto the needle. The animal was then pushed off the needle into the dish. In the technique used here, a pair of fine forceps was put into the mouth of the animal and the basal disc was grasped and pulled through the mouth. Both techniques can potentially damage the animals. However, the technique used here generally caused damage only in the peduncle or the hypostome. The area of damage due to Roudabush's technique is not mentioned; but his mortality rate of 30% is considerably greater than the less than 5% mortality rate of animals everted in this study. In the observation of histological sections of everted animals, areas with damaged or missing mesoglea are frequently encountered, and the area of "cell migration" described by Roudabush exactly fits the description of a damaged area. Figure 3 shows such a damaged area in a gastric piece and Figure 4 shows two sections of a damaged area in an animal 6 days after being everted. These pictures and many more like them are not evidence of cell migration but merely illustrate the normal role of the mesoglea in maintaining the structural integrity of hydra.

How then is it possible for so many animals to appear to stay everted when,

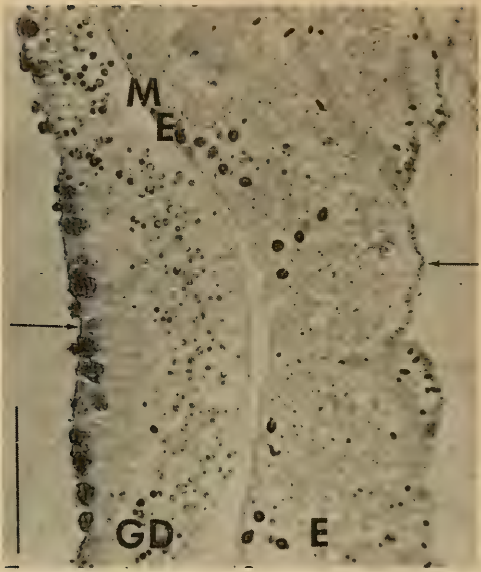

FIGURE 2. Section of an animal which is still everted after 24 hours. Note the presence of mucous droplets in both the epidermal and gastrodermal cells.

in fact, they managed to resume their normal body layer orientation? An additional group of animals was observed every 10 minutes for 5 hours and those which were not seen to re-turn were sectioned. Many of these had re-turned.

This experiment was then repeated with additional care. The gastrodermal

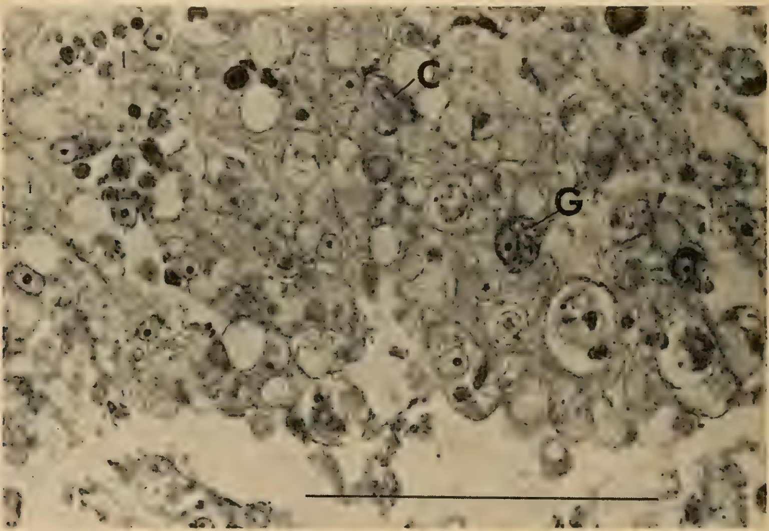

FIGURE 3. Damaged area in a hydra. Note the absence of mesoglea in this section and the absence of distinct cell layers.

cells of 30 hydra were stained by adding neutral red to the culture water. These animals were everted, placed in 5 dishes and observed continuously. When stained with neutral red an animal may be placed on a slide, covered with a coverslip and its cell layer orientation immediately determined, since principally the gastrodermal cells take up the stain. Figures 5 and 6 show the appearance of a normal and an everted animal stained with neutral red.

During the observation of the stained everted animals, any animal believed to have turned was immediately checked by the technique described above. In addition, after any unusual contraction, the animal was examined. All animals that were not prematurely removed from the dishes were observed to turn within 5 hours. Two methods of turning were observed. (1) Animals turned by the method described by Roudabush; turning by this method takes the animal several minutes to complete and is easy to observe. This method of re-turning is also the method generally described by Trembley, Ischikawa, and Weissman. (2) Other animals turned by a previously undescribed method. (Ischikawa does mention that some animals re-turn rapidly.) The everted animal was observed to contract into a tight toroidal shape and then to elongate. The maneuver appears normal and takes only a few seconds. But before contracting, the hydra is everted and after relaxing, the body layers of the hydra are oriented normally. This second method of re-turning is the one which was not observed earlier in this study and was not described by Roudabush. This fast turning method probably accounts for the many animals seen by Roudabush and seen in this

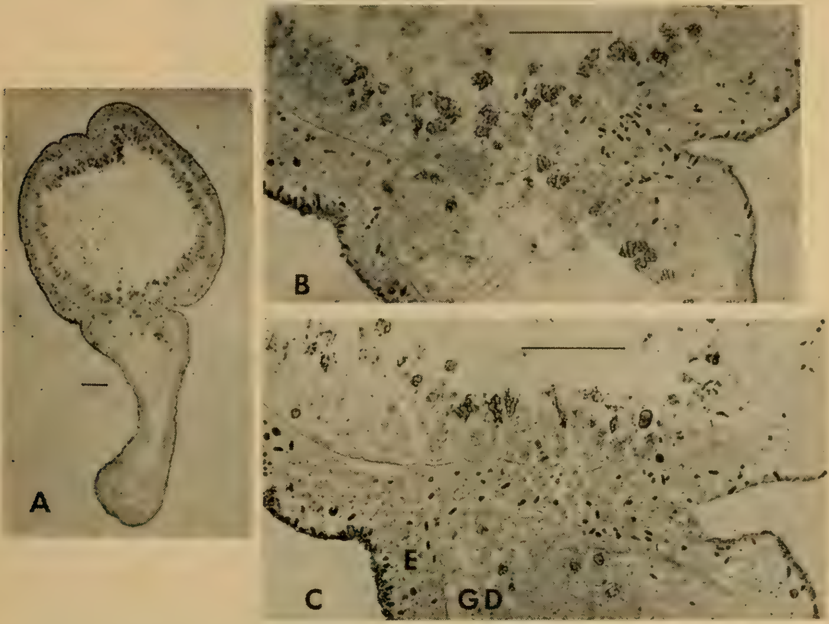

FIGURE 4. Damaged area in everted animal after 6 days showing mixing of cell layers. (A) and (B) are the same section and (C) shows the same area about 75 microns away. Animal was decapitated prior to everting.

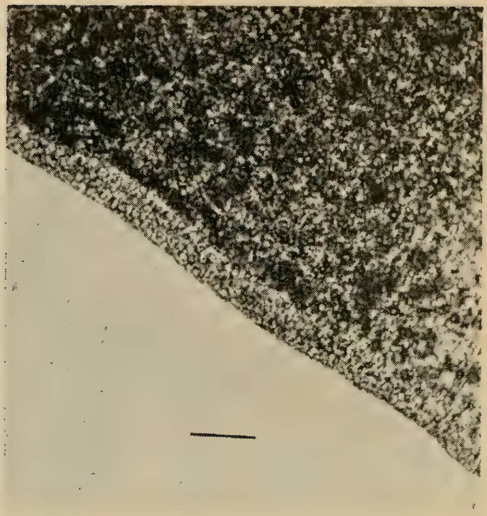

Figure 5. Whole mount of normal live animal stained with neutral red. Note the marked decrease in stain in the epidermis.

study with correctly oriented body layers but without any observation of their having turned back.

The two methods of re-turning are diagrammatically illustrated in Figure 7. On the left side of the figure the cell layer arrangement and location of tentacles for an everted animal is shown and a corresponding drawing on the right side of the figure shows the normal body configuration. In the center are drawings illustrating the two methods of re-turning. At the top the first or slow method is shown (Ia and Ib in Fig. 7) with the cell layers reversing by slow

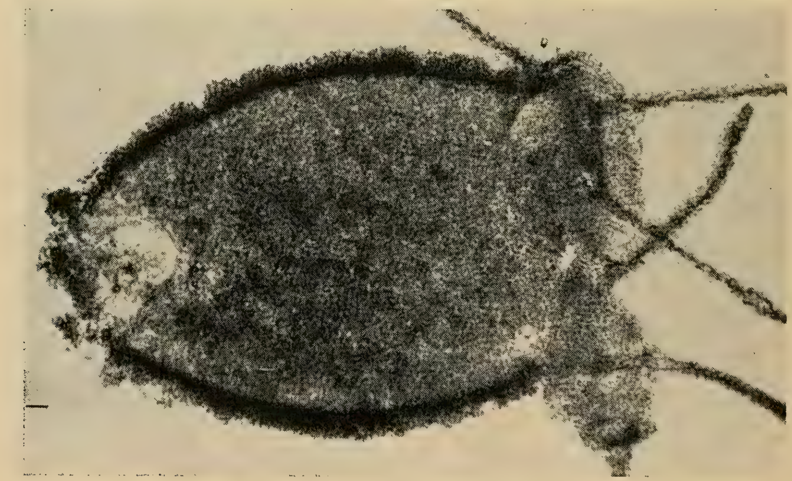

Figure 6. Whole mount of live bud, stained with neutral red, 5 minutes after everting. Note the dark border of gastrodermal cells. Damage to the basal disc can be clearly seen.

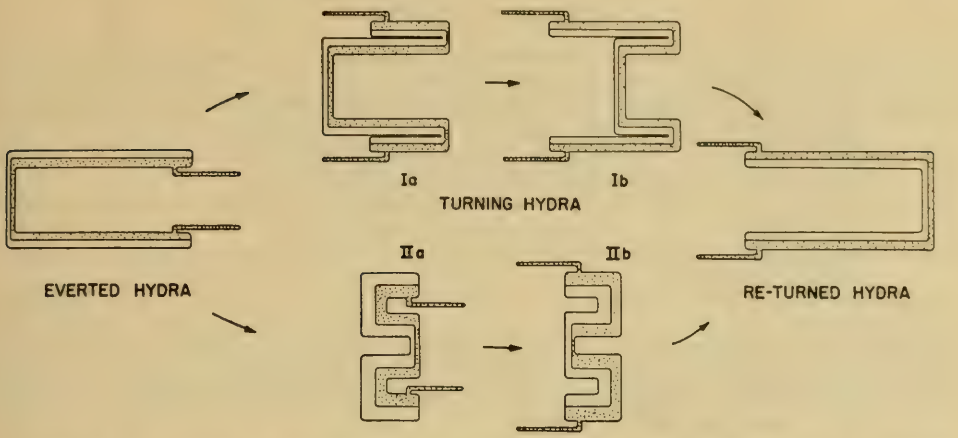

FIGURE 7. Schematic representation of methods of re-turning everted hydra. Everted hydra either peels back slowly (Ia, Ib); or they contract (IIa, IIb) and then relax to achieve the correct body layer orientation. All views are longitudinal sections with the epidermal layer stippled and tentacles shown.

migration of the body column over itself. This should be contrasted with the drawings at bottom center showing the second or fast method of turning. A hydra is schematically shown contracted into a tight toroidal shape just after contracting (IIa) and just prior to relaxing (IIb). By this maneuver of contracting from one direction (left to right in figure) and relaxing in the opposite direction the animal very quickly re-turns.

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SUMMARY

1. Hydra were everted by pulling their basal disc through their mouth and were then observed to determine the mechanism of regeneration. Animals that were not seen to re-turn to their original body layer orientation were fixed at time intervals from one-fourth to 144 hours and examined histologically. No evidence was found to support Roudabush's hypothesis that cell migration across the mesoglea could re-establish the normal body layer orientation. But areas damaged during everting were found which correspond with the regions of cell migration described by Roudabush.

2. The regeneration of everted hydra occurs solely as a result of re-turning by two methods: (a) slow re-turning resembling the turning of a sock when it is being removed; and (b) fast re-turning accomplished by a rapid contraction by the everted animal followed by relaxation to the re-turned or normal body layer orientation.

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GROWTH STIMULATION OF EMBRYONIC SPLEEN GRAFT BY ADULT CHICKEN SPLEEN ACROSS A MEMBRANE FILTER BARRIER

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The splenomegaly reaction observed in the chick embryo following the implantation of a piece of adult chicken on the chorioallantoic membrane (Danchakoff, 1916; Murphy, 1916; Willier, 1924) may be brought about by the proliferation of embryonic spleen cells in response to a stimulus from the adult spleen graft, or by the proliferation of adult donor cells following invasion and colonization in the host spleen. However, Biggs and Payne (1959) showed clearly that enlargement of the chick host spleen was due to the proliferation of both embryonic spleen cells as well as adult spleen cells. Four days after the intravenous injection of cockerel blood into 14-day chick embryos they were able to identify both male and female cells in almost equal proportions in the enlarged spleens of female hosts.

The response of embryonic spleen cells to stimulus from the adult spleen graft was elucidated in studies using metabolic inhibitors (Mun and Burns, 1964). When 5-fluorouracil was injected intravenously on the 11th day of incubation, two days after the implantation of adult chicken spleen on the chorioallantoic membrane, no enlargement of the host spleen was obtained. However, when the "inhibited" spleen was transferred to a new 10-day host, enlargement of the spleen of the new host was obtained. These experiments demonstrated clearly that although viable and competent adult spleen cells were present in the host spleen, they were not able to bring about enlargement of the host spleen by themselves. Were embryonic cells capable of responding to stimuli from the adult spleen cells necessary? To test this possibility a second series of experiments was conducted. Adult chicken spleen was implanted on the chorioallantoic membrane on the 9th day of incubation. 5-Fluorouracil was then injected intravenously on the 11th day of incubation and a piece of 17-day embryonic spleen was implanted on the 14th day of incubation. When the embryo was examined on the 18th day of incubation, we found that although the host spleen was again inhibited the embryonic spleen graft on the chorioallantoic membrane was distinctly enlarged. These observations suggest strongly that the splenomegaly reaction is due in part to the proliferation of embryonic spleen cells following stimulation by adult spleen cells.

We now ask: What is the nature of this stimulus? Can embryonic spleen cells be stimulated to proliferation in the absence of viable or competent adult spleen cells? Several attempts have been made to induce splenomegaly in the chick embryo by injecting cell-free homogenates or extracts of adult chicken spleen. Van Haeften (1957) implanted cell-free homogenates of adult chicken spleen, thymus, and muscle on the chorioallantoic membrane and observed a marked hypertrophy of the host spleen. Croisille (1958) injected intravenously cell-free extracts of adult chicken spleen into the chick embryo. Three injections were administered at 24-hour intervals beginning on the third day of incubation. He reported an increase of 25% in the size of the host spleen. DeLanney *et al.* (1962) injected the microsome fraction from adult chicken spleen into blood vessels in the chorioallantoic membrane of 9- and 11-day chick embryos and observed enlargement of the host spleen.

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In the above studies a degree of enlargement of the host spleen (25 to 30%) was obtained but it was slight when compared to the enlargement elicited by fresh adult chicken spleen tissue or viable cells (100 to 300%). The failure to obtain greater stimulation may be due to the limited amount of material which may be injected into the chick embryo at any one time. It may also depend on the particular stage of development of the host, *i.e.*, the particular injections may not have been administered at a critical stage of development. The use of membrane filter capsules containing viable and intact cells would appear to obviate some of these criticisms.

Mulnard (in DeLanney *et al.*, 1962) implanted membrane filter capsules containing fragments of adult chicken spleen on the chorioallantoic membrane on the 10th day of incubation. He observed an increase of 30% in the weight of the host spleen on the 17th day of incubation. However, in our hands, cells and tissue enclosed in membrane capsules which were implanted on the chorioallantoic membrane were usually found to be abnormal and undergoing degeneration when examined more than three days later (Mun and Ebert, unpublished).

In order to insure optimum growth of the grafted adult spleen tissue, we adopted a variation of the technique of parabiosis or synchorial anastomosis (Hasek *et al.*, 1958). Adult spleen tissue was grafted on the chorioallantoic membrane of a 9- or 10-day host embryo. This embryo was then joined to another embryo of the same age but was separated by a single membrane filter. Such a method would not only provide for better growth of the adult spleen graft, but would also expose a greater area of membrane (1 × 1 cm.) and permit diffusion of a larger amount of substance over a longer period of time (7 days).

MATERIALS AND METHODS

Fertile White Leghorn eggs or eggs from a cross between a Rhode Island Red male and a Barred Rock female were obtained from the University of Maine farms. The eggs were incubated at 37.5 to 38° C. for 9 or 10 days.

Because of the great fragility of blood vessels in the chorioallantoic membrane of the 10-day chick embryo, initial attempts to parabiose two embryos often resulted in hemorrhage and death of one or both of the pairs. The following technique was developed to reduce this mortality:

Two eggs of similar size were selected and placed side by side in a specially constructed holder which permitted both eggs to be rotated on their long axes. A window (10 × 12 mm.) was cut in each egg at the level where they come into contact. The shell and shell membrane were removed and the chorioallantoic membrane was permitted to drop. The cut edges of the shell and the shell membrane were coated with melted paraffin. A piece of Millipore[2] filter (type THWP 0.45 μ, 25 μ thick), Dacron mesh, or cotton gauze (15 × 20 mm.) was placed over the window of one egg and sealed with paraffin. This egg was then rotated slowly in the holder until the covered window was upside down and directly over the open window of the second egg. The two eggs were then joined together at the edge of the windows with paraffin. The joined eggs were placed in the incubator with the pointed ends down.

In control experiments the two eggs were separated by a piece of Dacron mesh, cotton gauze (15 × 15 mm.), or membrane filter (12 × 15 mm.) which was punctured with several small holes. In the experimental series a piece of adult chicken spleen was grafted on the chorioallantoic membrane of one parabiont before it was covered with the membrane filter. The tissue was obtained from chickens more than three months old and usually from a strain of chicken opposite to that of the host embryo. The adult spleen was removed, washed in sterile saline and cut into pieces two to four cubic millimeters.

TABLE I

Mean spleen/body weight of embryos joined with paraffin but separated by Dacron mesh, cotton gauze, or Millipore filter punctured with small holes

Treatment	No. cases	Mean spleen wt. (mg. ± S.D.)	Mean body wt. (gm. ± S.D.)	Log HS/BW $\times 10^4 \pm$ S.D.
Control				
with or without embryonic spleen graft	4	11.00 ± 1.70	18.32 ± 1.07	0.774 ± .051
Experimental				
1. parabiont without grafts	8	21.75 ± 17.89	13.56 ± 2.41	1.110 ± .242
2. parabiont with adult spleen graft	8	26.08 ± 11.76	14.01 ± 3.47	1.239 ± .170

The embryos were recovered on the 17th or 18th day of incubation. The host spleen was removed, blotted on filter paper and weighed to the nearest 0.2 mg. Because of differences in the age of the embryos at the time of operation as well as at recovery in the different experiments, the ratio of host spleen weight to body weight was computed. Cock and Simonsen (1958) reported a positive correlation between host spleen and body weights in control series. Because the variance of the spleen weights also increased with the mean, a $\log_{10}$ transformation (Payne and Jaffe, 1962) was used before applying Student's T-test to compare weights between control and experimental groups.

RESULTS

In the first series of experiments, a 10-day chick embryo host with an adult chicken spleen graft on the chorioallantoic membrane was joined to another 10-day embryo but separated by a piece of Dacron mesh, cotton gauze, or membrane filter which had been punctured with many small holes. After seven more days of incubation, the host spleens in both the parabiont with the adult chicken spleen graft as well as its partner were significantly larger ($p > .05$) than the host spleens in control pairs without adult chicken spleen grafts (Table I).

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[2] Millipore Filter Corporation, Bedford, Mass.

Table II

Mean spleen/body weight of embryos joined with paraffin but separated by Millipore filter membrane

Treatment	No. cases	Mean spleen wt. (mg. ± S.D.)	Mean body wt. (gm. ± S.D.)	Log HS/BW × 10⁴ ± S.D.
Control				
with or without embryonic spleen graft	11	13.09 ± 3.55	14.99 ± 3.65	1.011 ± .292
Experimental				
1. parabiont without grafts	12	8.10 ± 3.07	9.60 ± 3.18	0.922 ± .156
2. parabiont with adult spleen graft	12	19.44 ± 7.26	9.58 ± 3.38	1.301 ± .138

When the embryo containing the adult chicken spleen graft was separated from its partner by a piece of intact membrane filter, although the spleen of the embryo with the adult chicken spleen graft was greatly enlarged ($p > .05$) the weight of the spleen of its partner was not different from that in control pairs without the adult chicken spleen graft (Table II).

Because the failure to obtain enlargement of the host spleen may be due to the distance between the adult spleen graft in one embryo and the host spleen of its partner, as well as the poor contact between the outermost layer of the chorioallantoic membrane and the membrane filter, a second series of experiments was conducted in which a piece of embryonic spleen tissue was either placed on the top of the membrane filter, opposite the adult spleen graft, or implanted directly on the chorioallantoic membrane of the second embryo before it was rotated and joined with the first embryo. Embryonic spleen tissues were obtained from 14- to 17-day chick embryos. Spleen tissue at this particular stage of development was selected because in earlier studies using the chorioallantoic membrane grafting technique we were not able to detect any ability to induce splenomegaly until one to two weeks after hatching (Mun, *et al.,* 1962). The embryonic spleen weighing 7 to 12 mg. was cut in half or implanted whole. Seven to 8 days after operation, both adult and embryo spleen grafts were cut out from the chorioallantoic membrane, trimmed, blotted on paper, and weighed (Mun and Burns, 1964). The host spleens were also removed and weighed. Table III shows that the host spleen in embryos with

Table III

Mean embryonic and adult spleen graft weight and host spleen/body weight of embryo pairs separated by unwashed Millipore filter membrane

Treatment	No. cases	Graft wt. (mg. ± S.D.)	Mean spleen wt. (mg. ± S.D.)	Mean body wt. (gm. ± S.D.)	Log HS/BW × 10⁴ ± S.D.
Control					
with 14–17-day embryo spleen grafts	25	19.68 ± 10.09	11.58 ± 3.66	16.59 ± 3.93	.834 ± .106
Experimental					
1. parabiont with 14–17-day embryo spleen graft	39	30.63 ± 14.88	12.21 ± 4.15	16.76 ± 4.62	0.856 ± 0.111
2. parabiont with adult spleen graft	39	73.29 ± 40.53	57.28 ± 37.39	16.42 ± 4.97	1.470 ± 0.526

the adult spleen graft was strikingly enlarged but the spleen of its partner, separated by the filter, was not enlarged. However, when the embryonic spleen graft on the chorioallantoic membrane of this partner was examined, it was found to be significantly larger than that in control embryos, *i.e.,* pairs which had only embryonic spleen grafts but no adult spleen grafts ($p > .01$). In eleven of a total of 76 pairs examined in this experiment, one or both of the grafts on the chorioallantoic membrane was "partially successful" or "not succesful" (Mun *et al.,* 1959). These cases were not included in Table III.

Because of a recent report by Cahn (1967) that membrane filters from several manufacturers contain detergents, a fact not contained in descriptive brochures, a third series of experiments was conducted in which the filters were washed in a Seitz filter holder with 100 cc. of hot distilled water and rinsed with cold sterile saline. In previous experiments the filters were simply soaked in or moistened with cold sterile saline before use.

TABLE IV

Mean embryonic and adult spleen graft weight and host spleen/body weight of embryo pairs separated by washed Millipore filter membrane

Treatment	No. cases	Mean graft wt. (mg. ± S.D.)	Mean host spleen wt. (mg. ± S.D.)	Mean body wt. (gm. ± S.D.)	Mean spleen wt. Log HS/BW × 10^4 ± S.D.
Control					
with 14–17-day embryo spleen graft	11	22.82 ± 4.88	13.02 ± 1.97	15.91 ± 2.13	.912 ± .089
Experimental					
1. parabiont with 14–17-day embryo spleen graft	16	43.84 ± 16.91	15.90 ± 3.94	17.31 ± 2.71	.957 ± 0.074
2. parabiont with adult spleen graft	16	80.48 ± 17.56	50.76 ± 13.83	16.33 ± 2.05	1.477 ± .114

When the embryonic spleen graft was separated from the parabiont with an adult chicken spleen by a washed membrane filter, a greater proportion of both embryonic and adult spleen grafts in contact with the washed membrane filter were pink and well vascularized, or "successful." The mean weight of the embryonic spleen grafts was significantly greater ($p > .001$) than that in control pairs without adult chicken spleen grafts. The mean weight of the host spleen of the partner containing the enlarged embryonic spleen graft was not different from that of control pairs without adult chicken spleen grafts (Table IV [3]).

DISCUSSION

Auerbach and Globerson (1966) reported the ability of adult mouse spleen cells to induce "splenomegaly" in allogeneic spleen cells across an intervening membrane filter barrier *in vitro.* By counting the number of sections/culture as well as measuring the surface area/section in histological preparations, they noted "splenomegaly" in 6/8 cultures. In these studies we observed an enlargement of the embryonic spleen graft separated from an adult spleen graft by a membrane filter barrier. However no enlargement of the spleen of the host with the embryonic spleen graft was observed. This observation argues strongly against the possibility that intact adult spleen cells were able to traverse the barrier. We have shown earlier that embryonic spleen grafts, placed adjacent to adult spleen grafts, acquired the ability to elicit splenomegaly in new hosts as soon as 2–3 days (Mun *et al.,* 1962). The embryos in these experiments were joined for at least 7 days, and the enlarged spleen, if it did contain immunologically competent cells, would have had sufficient time to elicit splenomegaly in the host spleen.

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[3] Taken in part from data submitted by Miss Longo to the Department of Zoology, University of Maine, in partial fulfillment of the requirements for the Bachelor of Arts degree.

McBride *et al.* (1966) observed that 17-day chick embryos which had received immunologically competent cells from an adult attained a higher degree of immunological competence per unit of cells within 2 days. Thus there was an "accelerated maturation" of the embryonic cells. However, in these studies, although the embryonic spleen grafts were enlarged, the failure to obtain enlargement of the host spleen in the same embryo argues against the possibility that proliferation of embryonic spleen cells in the graft on the chorioallantoic membrane was accompanied by accelerated immunological maturation.

Thus the observed differences in weights of the host spleen and embryonic spleen grafts may be due to differences in their response to stimulating substances from the adult spleen graft. Several possibilities may be advanced: First, we may consider the difference in the distance between the adult spleen graft and the embryo spleen graft, or host spleen. In most cases, the embryonic spleen grafts were found adhering to or near the membrane filter and well incorporated in the chorioallantoic membrane. However, in at least two cases, the embryonic spleen grafts were found, at the time of recovery, more than 1 cm. away from the membrane filter area. In both cases the embryonic spleen grafts were distinctly enlarged. These observations strongly suggest that the substances capable of stimulating proliferation of embryonic spleen cells were disseminated *via* the vascular system. Thus the differences observed may not be due solely to the greater distance between the adult spleen graft and the host embryo spleen.

A second possibility which we may consider is the difference in age between the host spleen and the embryonic spleen graft. The host spleens were 9 or 10 days old at the time of operation but the embryonic spleen grafts were obtained from older 14- to 17-day-old chick embryos. In a recent study we showed clearly that spleen or thymus tissues from older 14- to 17-day-old embryos were better able to induce immunological tolerance than tissues from younger 4- to 10-day embryos (Mun *et al.,* 1967). Thus the differences in response to the stimulating influences of the adult spleen graft may be due to differences in the age or stage of development between the embryonic spleen graft and the host spleen. The mechanism of control of differentiation or proliferation remains to be further elucidated.

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SUMMARY

1. In an attempt to further analyze the sequence of steps resulting in homologous splenomegaly in the chick embryo, the following series of experiments were conducted to elucidate, in particular, the role of the host spleen component in the absence of intact adult donor spleen cells. In the first series of experiments two 10-day chick embryos were joined together with paraffin but separated by cotton gauze, Dacron mesh or a piece of membrane filter which was punctured with many small holes. Spleen enlargement in both host embryos was observed when a piece of adult chicken spleen was implanted on the chorioallantoic membrane of one of the pair. When the two embryos were separated by an intact piece of membrane filter (Millipore THWP 0.45 μ, 25 μ thick) no enlargement was observed in the spleen of the embryo without the adult spleen graft. Because of the possibility that the contact between the chorioallantoic membrane and the membrane filter was not adequate, in order to encourage vascularization, a piece of 17-day embryonic spleen tissue was grafted on the chorioallantoic membrane of one embryo. This was joined with another embryo containing an adult chicken spleen graft. The two grafts as well as the two embryos were separated by a piece of membrane filter. Although enlargement of the spleen of the host with the embryonic spleen graft was not obtained, a striking enlargement of the embryonic spleen graft was elicited. Similar results were obtained when the membrane filter was washed with more than 100 cc. hot distilled water, but the number of poor or unsuccessful grafts was sharply reduced.

2. These experiments reveal a differential response of spleen tissue from 17-day embryos and 10-day embryos to substances which are able to diffuse through the membrane filter from the adult spleen graft.

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SELECTIVE FEEDING ON ALGAL CELLS BY THE VELIGER LARVAE OF NASSARIUS OBSOLETUS (GASTROPODA, PROSOBRANCHIA)[1, 2]

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Food is an important factor determining the survival and developmental rate of planktotrophic larvae. Both the quality and quantity of food available to molluscan veligers have been related to their successful completion of pelagic life. The nutritional value of various species of algae to certain bivalve larvae (*viz., Venus mercenaria, Crassostrea virginica, Ostrea edulis,* and *Mytilus edulis*) has been studied by Davis (1953), Davis and Guillard (1958), Walne (1963), Davis and Calabrese (1964), and Bayne (1965). However, while it is known from these studies that some species of algae are apparently more favorable as food than others, no experiments have yet been made that test the ability of molluscan larvae to discriminate between phytoplanktonic forms or that demonstrate that veligers can feed selectively upon those microorganisms that result in faster growth. Only the single experiment described in a short note by Loosanoff *et al.* (1953, pp. 586–587) declares that ". . . the larvae [of *Venus mercenaria*] showed both quantitative and qualitative selectivity in feeding . . . qualitative selectivity of food was demonstrated when the larvae were fed a mixture of several food organisms; given a mixture of *Porphyridium* (3 μ) and *Chlamydomonas* (10 μ), the larvae rejected *Porphyridium* while ingesting the much larger *Chlamydomonas*." No further details of this experiment are given. The question as to whether a gastropod larva can choose or select from a mixture of microorganisms remains to be answered.

The method of feeding by gastropod veligers has been described by Werner (1955) for the larvae of *Crepidula fornicata* and by Thompson (1959) for those of the nudibranch *Adalaria proxima*. Feeding is accomplished by the velum. According to Thompson (p. 241), "the long velar cilia serve a dual purpose in both imparting a forward motion to the larva and bringing a constantly renewed supply of seawater within the influence of the feeding apparatus." A second row of cilia on the hind side of the velum serves in the actual collection and propulsion of the food particles. The direction of beat of these cilia is such that food from either velar lobe is directed toward the mouth (*vide* Werner, 1955, Fig. 9, p. 196). The border of the mouth is ciliated and particles that are sufficiently small are carried into the foregut. Material which is too large or rejected for other reasons is carried forward along the foot to its border where it is cast off. This pattern of feeding is common to all gastropod veliger larvae where it has been observed. The differences

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in detail among various species are mainly concerned with the rejection tracts along the foot. Our studies have confirmed this mode of feeding in *Nassarius obsoletus*.

If veliger larvae are allowed to feed on a mixture of several species of algae, the total or absolute amount consumed at the end of a given time may be determined by finding the difference between concentration of the resulting mixture and that of a control identical in all ways but not containing larvae. If the veligers feed at random, the relative proportions of the different species of algae should be the same in both mixtures at the end of the experiment. However, if larvae feed preferentially on any one species, this will be indicated by a shift in the relative proportions of the different algae compared with that found in the control mixture. Each species of alga can be assigned a rank preference according to the proportion eaten. We will present here evidence that the veliger larvae of the prosobranch gastropod *Nassarius obsoletus* can feed selectively when offered a mixture of three species of algae.

MATERIALS AND METHODS

The larval development of *Nassarius obsoletus* and the methods for rearing the veligers have already been described (Scheltema, 1962). Larvae were "hatched" in the laboratory from egg capsules collected from Barnstable Harbor (Cape Cod) during July and August. The eggs were stored at around 12° C. to inhibit development until needed in the experiments (*vide* Scheltema, 1967). When transferred from a storage temperature of 12° to a room temperature of approximately 25° C., the egg capsules released larvae in from several hours to three or four days. The veligers were raised in 10-liter culture jars at room temperature. Following a change in sea water every third or fourth day, *Phaeodactylum tricornutum* was added to each culture so that the resulting concentration of algal cells was approximately 2×10^5 cells/ml. Each 10-liter culture contained several thousand larvae. Within two weeks from the time of "hatching" the veligers reached the settling stage, at which time some metamorphosed larvae were observed on the sides of the culture jars. The veligers grew from approximately $250\,\mu$ to $600\,\mu$ during the free-swimming larval period.

Two large uni-algal cultures of *Phaeodactylum* in 15-liter carboys were maintained on an autoclaved medium of a commercial chemical garden fertilizer. These cultures served as the source of food for both feeding experiments and rearing the larvae. Cultures of *Cyclotella nana* and *Dunaliella tertiolecta,* used for the feeding experiments, were grown axenically in 1- and 2-liter flasks on "f/2" medium (Guillard and Ryther, 1962). All cultures were illuminated continuously in a constant temperature room at 16° C. The density of the cultures before use for experimentation was from 4×10^5 to 10×10^5 cells/ml. of *Cyclotella,* from 8×10^5 to 20×10^5 cells/ml. of *Dunaliella,* and from 15×10^5 to 40×10^5 cells/ml. of *Phaeodactylum.*

Feeding experiments were conducted in 3-liter jars at room temperature. Suspensions of phytoplankton to be used were made up by the addition of known amounts of the culture described above to filtered sea water so that the resulting concentration was about 3×10^5 cells/ml. One to $1\frac{1}{2}$ liters of cell suspension were added to each of several 3-liter jars. Larvae which had been starved for six hours prior to the experiments were added to each jar except that which was to serve as a control. Each experimental jar contained from several hundred to several thousand veligers of the same age. The mixtures were stirred and aerated continuously for a period of from 10 to 16 hours. At the end of this period the concentrations of each species of algae in both the control and the experimental jars were determined. This was done by obtaining a sample of each with a wire loop having an 0.5-mm. diameter, fixing the sample with an iodine-acetic acid solution, and making counts on a Levy phase hemacytometer using a phase microscope at a magnification of 250 ×. The entire grid of the hemacytometer was used. Each mixture was counted several times; the total number of cells counted from each control was *ca.* 1500 cells. From each of the experimental mixtures, between 500 and 1500 cells were enumerated. It required 4 to 7 counts to achieve this total. The standard errors of the estimates ranged between 4 and 12% of the mean.

TABLE I

*Preference among three different species of algal food by veliger larvae of Nassarius obsoletus (Say) (based on differences in numbers of cells ingested)**

Species of algae	Percentage of experiments		
	1st choice	2nd choice	3rd choice
Cyclotella	*81.3*	18.8	0.0
Phaeodactylum	12.5	*62.5*	25.0
Dunaliella	6.3	18.8	*75.0*

* Results from sixteen experiments.

RESULTS

The relative proportions of the three species of algae in the experimental mixtures differed significantly from those of the controls. Each of the three species of algae could be assigned a rank of preference according to the percentage of cells eaten. If all the data are pooled and summarized, *Cyclotella* was the first choice in 81.3% of all the experiments. In most instances *Phaeodactylum* was second choice while *Dunaliella* most commonly was last to be selected (Table I).

A triangular graph with three coordinate axes can be used to compare control and experimental concentrations in the experiments (Figs. 1, 2, 3). The coordinate axes are the three perpendicular bisectors, and are labelled at the vertices, "P", "D", and "C" for *Phaeodactylum, Dunaliella,* and *Cyclotella,* respectively. The scale runs from 100% at the vertex to 0% at the side opposite the vertex. A point at the intersection of the three bisectors represents a mixture in which each of the three species make up 33.3% of the total, that is, each is present in equal amounts. Points indicated by squares represent the compositions of the control while points shown by circles represent the compositions of the experimental mixtures. Lines connect experiments with the controls to which they are compared; control points are lettered and experimental points numbered so that each line is labelled with a letter and number. The experiments were distributed among three veliger size-categories so that it is possible for experiments having a common control to be represented on different graphs if the experiments involve veligers of different ages. Each graph represents veligers in the same size-category. The size-categories represent different ages that roughly correspond with the three developmental stages that have been described previously (Scheltema, 1962, p. 4, Pl. I) except that the category in Figure 2 here overlaps the "early" and "intermediate" stages. Newly "hatched" veligers, those that had not fed on plankton prior to the experiment, are grouped separately (Fig. 1).

Dunaliella is rarely the first choice of veligers of any size. In all but one case (D-1, Fig. 1), the percentage composition of *Dunaliella* is greater in the experimental mixture than in the control. On the graphs this is indicated by the fact that experimental points are closer to the *Dunaliella* vertex ("D") than the control points. This becomes more marked with increasing size of the larvae.

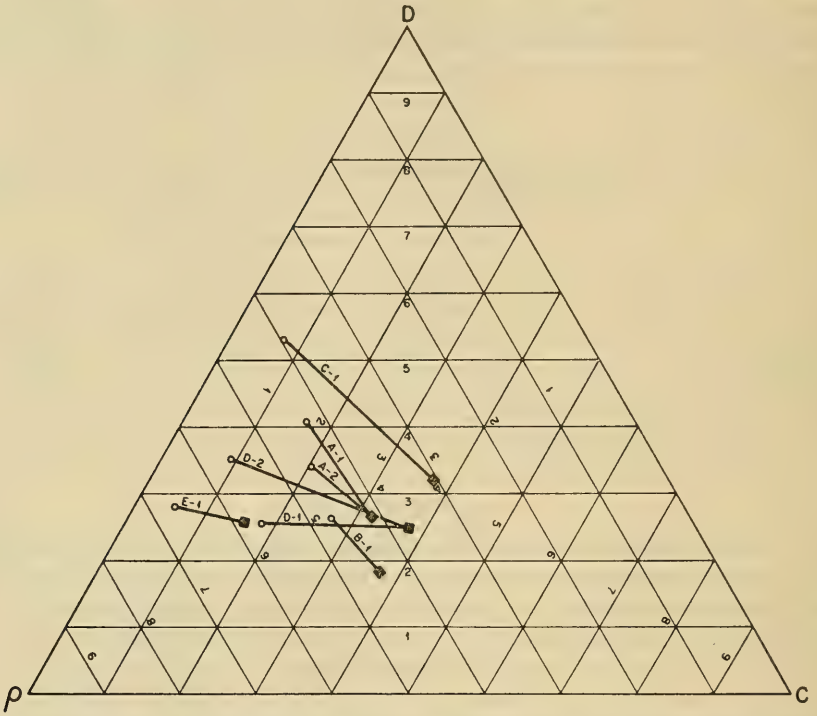

FIGURE 1. Choice among three different species of algal food by newly emerged veliger larvae of the gastropod *Nassarius obsoletus* (Say). For explanation see text.

Experimental points are always further from the *Cyclotella* vertex ("C") than points representing controls. This shows that the percentage of *Cyclotella* remaining in the mixture always decreases and consequently that *Cyclotella* has been consumed by the larvae. *Cyclotella* is never the last choice of veligers of any size.

Preference for *Phaeodactylum* is different for newly "hatched" than for older larvae. Figure 1 shows that the percentage composition of *Phaeodactylum* is greater in the experimental mixture than in the controls, while Figures 2 and 3 show the experimental composition of *Phaeodactylum* in per cent to be less than that of the control. Older larvae have a greater preference for *Phaeodactylum* than do newly hatched larvae.

Although no marked difference in the outcome of the experiments can be related to the original proportions of the three algal species present, future experiments should use initial mixtures of varied composition.

The results shown on the triangular graphs (Figs. 1, 2, 3) can be compiled and summarized in a somewhat different way. The relative amount of each algal species removed in the experiments can be computed. Hence:

$$\%\ \text{removed} = \frac{\text{control concentration—experiment concentration}}{\text{control concentration}} \times 100$$

When the data from all the experiments in each category are averaged the results (Table II) show that *Cyclotella* is preferred over *Dunaliella* by all size categories of larvae ($P \cong 0.05$).

TABLE II

Average percentage of cells removed from suspension by veliger larvae of Nassarius obsoletus at different stages of development when given a choice of three algal species as food

Stage of larval development	Species of algae		
	Cyclotella	*Phaeodactylum*	*Dunaliella*
	Average per cent removed*		
Newly emerged veliger larvae (6 experiments)	72.9 ± 6.6	39.2 ± 8.1	33.1 ± 8.4
Veliger larvae of 200–400 μ length (7 experiments)	83.9 ± 3.8	69.1 ± 8.8	50.1 ± 11.5
Veliger larvae of 400–600 μ length (3 experiments)	70.2 ± 11.9	71.9 ± 4.0	24.5 ± 3.6

* One standard error of the mean indicated.

In order to check the possibility that rearing larvae upon *Phaeodactylum* might influence the veliger's behavior in the feeding experiments, two choice experiments were made using two-day-old veligers that had been exclusively reared on either *Dunaliella* or *Cyclotella*. The outcome of the experiments was conclusive. Veligers reared on *Dunaliella* consumed 60% *Cyclotella*, 50% *Phaeodactylum* and 21% *Dunaliella*. Veligers reared on *Cyclotella* ate 66% *Cyclotella*, 67% *Phaeodactylum*, and 31% *Dunaliella*. Selection against *Dunaliella* occurred regardless of the species of microorganisms upon which the larvae had been previously reared.

Newly hatched larvae do not discriminate between *Phaeodactylum* and *Dunaliella*; intermediate stage larvae show no clear-cut preference for *Phaeodactylum* over either *Dunaliella* or *Cyclotella*. The largest size class of veligers show equal preference for *Phaeodactylum* and *Cyclotella*. The net result is an increasing preference for *Phaeodactylum* with increasing veliger size.

The relative volumes consumed in each of the experiments may be computed if the cell volume for each species of alga is known. The average volume for *Cyclotella* may be regarded as approximately 203 μ^3 (Guillard and Cassie, 1963, p. 62), that of *Phaeodactylum* and *Dunaliella* as 120 μ^3 and 400 μ^3, respectively (Parsons *et al.,* 1961, p. 1004). If the volume of *Cyclotella* cells consumed is arbitrarily given a value of one, then the relative volume for the other two algal species for each size range of veliger larvae may be computed from the data in Table III. These values show that although *Dunaliella* cells are removed from suspension at a significantly slower rate, the volumes of cells removed by all veligers up to 400 μ in length are nearly identical to that of *Cyclotella.* Only in larvae over 400 μ does the relative volume of *Dunaliella* removed become somewhat less. At all ages the relative volume of *Dunaliella* removed by veligers seems to equal or exceed that of *Phaeodactylum.*

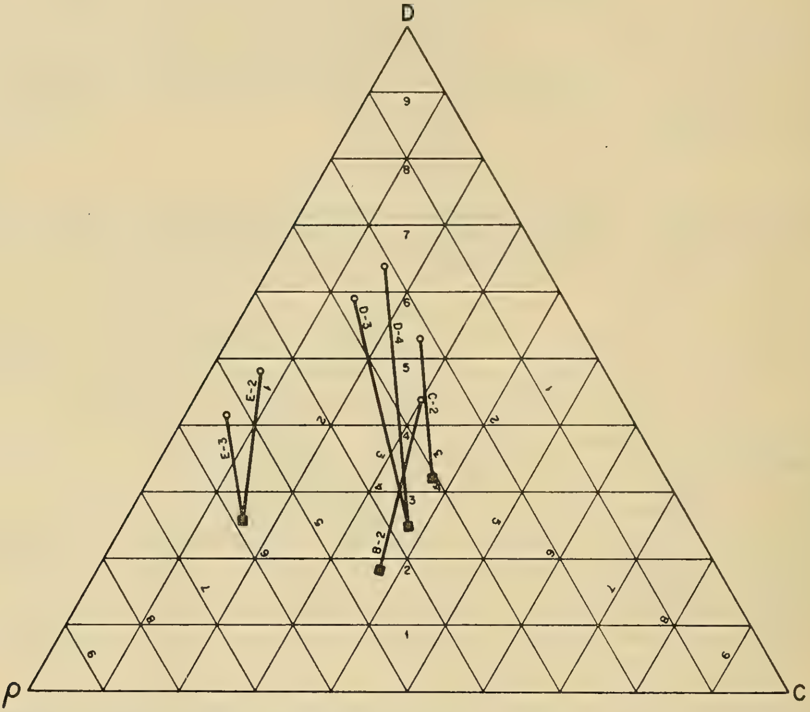

FIGURE 2. Choice among three different species of algal food by veliger larvae of the gastropod *Nassarius obsoletus* with a shell length between 250 μ and 400 μ. For explanation see text.

Discusssion

Three qualities of microorganisms in the natural environment are likely to make possible their selection as food by larvae. These are their (1) size, (2) availability or concentration, and (3) chemotactile attractiveness or "taste."

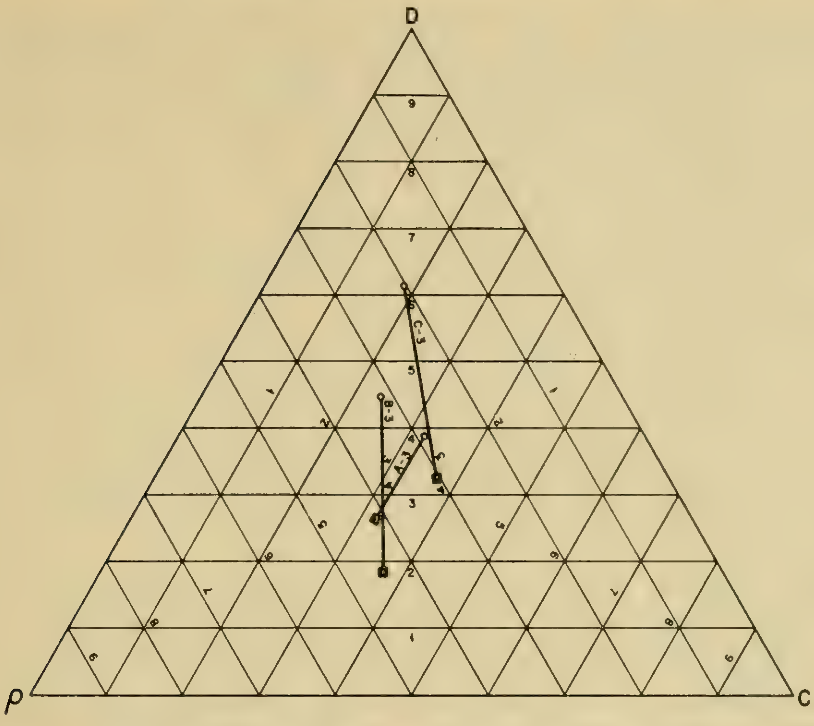

FIGURE 3. Choice among three different species of algal food by veliger larvae of the gastropod *Nassarius obsoletus* with a shell length between 400 μ and 600 μ (creeping-swimming stage). For explanation see text.

The size of the microorganisms which may be ingested is limited by the diameter of the mouth and intestine of the larvae. Thorson (1946, p. 447) summarizes data on the diameter of the esophagus and anterior portion of the intestine

TABLE III

Relative volume of cells consumed by veliger larvae of Nassarius obsoletus when given a choice of three algal species as food

Stage of larval development	Species of algae		
	Cyclotella	*Phaeodactylum*	*Dunaliella*
	Relative volume removed		
Newly emerged veliger larvae (6 experiments)	1.0	0.3	0.9
Veliger larvae of 200–400 μ length (7 experiments)	1.0	0.5	1.2
Veliger larvae of 400–600 μ length (3 experiments)	1.0	0.6	0.7

of prosobranch veligers and concludes that the theoretical size range of food must be from 5 to 45 μ. The size range of food actually accepted falls within these dimensions but is usually less than 30 μ. Hence, prosobranch veliger larvae are largely restricted to nannoplanktonic microorganisms (< 25 μ diameter) as food and in nature consequently must be restricted to dinoflagellates, minute species of diatoms and possibly bacteria. It may be that the shape of some food organisms affects their acceptability.

It is rather well known that when larvae of Mollusca are placed in very dense concentrations of algae, their ability to feed becomes impaired. For example, Loosanoff *et al.* (1953) showed that *Venus mercenaria* larvae held in heavier than optimum concentrations of cells contained less food in their stomachs than those held in lighter food concentrations. Veligers of *Nassarius obsoletus* when placed in very heavy concentrations of algal cells are unable to feed normally and reject cells along the foot. The concentrations used in the experiments here described were within the range at which cells were readily accepted and near those concentrations observed in regions where the larvae regularly occur naturally. Hence Hulburt (1956) showed 10^6 to 10^7 cells/1. total phytoplankton in Great Pond, Massachusetts, values that compare reasonably well with *ca.* 10^5 cells/ml. used by us.

The experiments do not reveal how the larvae select their choice of food, whether it be by size, concentration or chemotactile sense. However, they do show that a direct comparison of the "food value" of different algal species must take into account the volume or biomass of cells consumed per unit time, and that this varies according to the phytoplankton species encountered.

SUMMARY

Planktotrophic veliger larvae of the prosobranch gastropod *Nassarius obsoletus* (Say) are able to feed selectively. Presented with a mixture of *Dunaliella tertiolecta, Phaeodactylum tricornutum,* and *Cyclotella nana,* they exhibit a definite order of preference in the number of cells of each algal species they ingest. *Dunaliella* is clearly the last choice for veligers of all sizes. Early larvae choose *Cyclotella* in preference to the other two species, but with increasing veliger size and age, ability to discriminate between *Phaeodactylum* and *Cyclotella* diminishes while the selection of *Phaeodactylum* in preference to *Dunaliella* increases. However, in recently emerged and intermediate stage veliger larvae the relative cell volume of *Dunaliella* consumed is approximately equal to that of *Cyclotella,* whereas that of *Phaeodactylum* is substantially less than that of either of the other two algal species. In older larvae the relative volume of *Cyclotella* eaten exceeds the volumes of both other species, whereas the volumes of *Phaeodactylum* and *Dunaliella* become approximately equal.

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A PHEROMONE-LIKE DISPERSANT AFFECTING THE LOCAL DISTRIBUTION OF THE EUROPEAN HOUSE CRICKET, ACHETA DOMESTICA

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The use of visual and auditory cues by animals to promote the aggregation or dispersion of conspecifics is well known, and mounting evidence clearly outlines the important role that chemical stimuli have along the same lines. The present paper reports upon the interaction of an attractant system with a repellant one, which together appear to aid in the regulation of local population density in the European house cricket, *Acheta domestica.* The repellant system, at least, has certain pheromonal characteristics.

METHOD

Adult crickets were obtained from a commercial dealer and held in 37 × 90 × 35 cm. glass-sided community terraria until needed. Chick mash, water and wooden blocks were provided at all times. The crickets perched on the sides and tops of the blocks.

The activity measured was the movement of crickets up onto control and experimental vertical wooden blocks in test terraria. These blocks were 4 × 4 cm. along each side, but with heights of either 10 or 16 cm. Experimental blocks were conditioned by placing them in the community cages containing 200–500 crickets or in smaller terraria holding known numbers of crickets. During this treatment, the experimental blocks became stained with feces and other products. The control blocks were identical to the experimental except that they were never exposed to the crickets.

The experiments were performed in 6-quart (15 × 24 × 16 cm.) or 5-gallon (21 × 30 × 25 cm.) glass test terraria covered with glass tops; chick mash and water were provided. Room temperature was 28° ± 2° C. Heavy cardboard covered the laboratory windows and the four centrally located, incandescent room lights were on an automatic 12-hour light cycle.

On the afternoon preceding each experiment the requisite numbers and sex of crickets were placed in the test terraria to permit them to explore the cage and to begin to establish social hierarchies. The next morning control and experimental blocks were introduced into each cage, with randomized placement of the blocks at two pre-arranged sites. Thereafter, the distribution of the crickets relative to the blocks or cage floor was recorded at predetermined intervals. In certain experiments the blocks were never changed in position, but in others, after each reading the crickets were chased from their previous location in the cage and the blocks were repositioned randomly between the two sites.

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RESULTS

Series 1

These experiments were designed to test the reactions of 24 single males and of 12 social groups (2 males and 2 females) toward the control and experimental blocks. Two 10-cm. blocks were used per 6-quart terrarium; the observa- The blocks were not repositioned after each reading. The results of the first 90 minutes of exposure of single males (Fig. 1a) showed that the experimental structures were selected most frequently. There was a rapid movement onto the exposed blocks during the first five minutes, followed by a period of readjustment for the next 15 to 25 minutes. Thereafter, the distribution of crickets became stabilized with about 18 on the experimental blocks, 1 on the controls and 5 on the cage floor. The experimental and control structures each comprised 28% of the area avaialable for crickets. Hence, one would expect an even distribution of animals on the two types of blocks if there were no cues to attract or repel them. Even though chi-square tests were not run because randomization of block position and displacement of the crickets did not occur after each reading, it is clear that the single males are responding either positively to those structures previously exposed to crickets or negatively to the control structures. The same behavior held throughout the 31-hour period for single males and for the social groups as well (Fig. 1b).

Series 2

These experiments were designed to test the hypothesis that the crickets in series 1 were repelled by the control blocks and not attracted by the experimental ones. Four male and four female crickets were introduced into each of four 5-gallon terraria. Two individually marked control 16 cm. blocks were introduced into each, and a record of the location of the crickets was taken at intervals of two hours or more for a total of five readings. After each reading the crickets tions of the cricket distribution began immediately and continued for 31 hours. were chased from their position, and the blocks were repositioned randomly. Summing all readings, a total of 58 observations (72%) of males on control blocks were made out of a possible 80. Comparable figures for females were 64 observations out of 80 possible (80%). These percentages are comparable to those obtained in series 1 where about 75% of the males were on the experimental blocks and 4% on the controls, while for females the percentages are 91 and 0, respectively. It seems that a relatively high and constant percentage of crickets ascend blocks, and the hypothesis that the crickets were repelled by some feature of the control blocks in series 1 was not supported.

Series 3

Series 1 and 2 suggest that crickets were responding positively to some characteristic of the experimental blocks, and the next experiments were designed to distinguish between certain types of chemical cues and other cues as the attracting factor. Five 5-gallon terraria, each with four males and four females, were set up, and experimental and control blocks were introduced. Both blocks were covered with one unit of tissue paper ("Kleenex") at the start of the experiment (but after the experimental blocks had been conditioned in the community cage). If the factor attracting the crickets were mobile or somehow

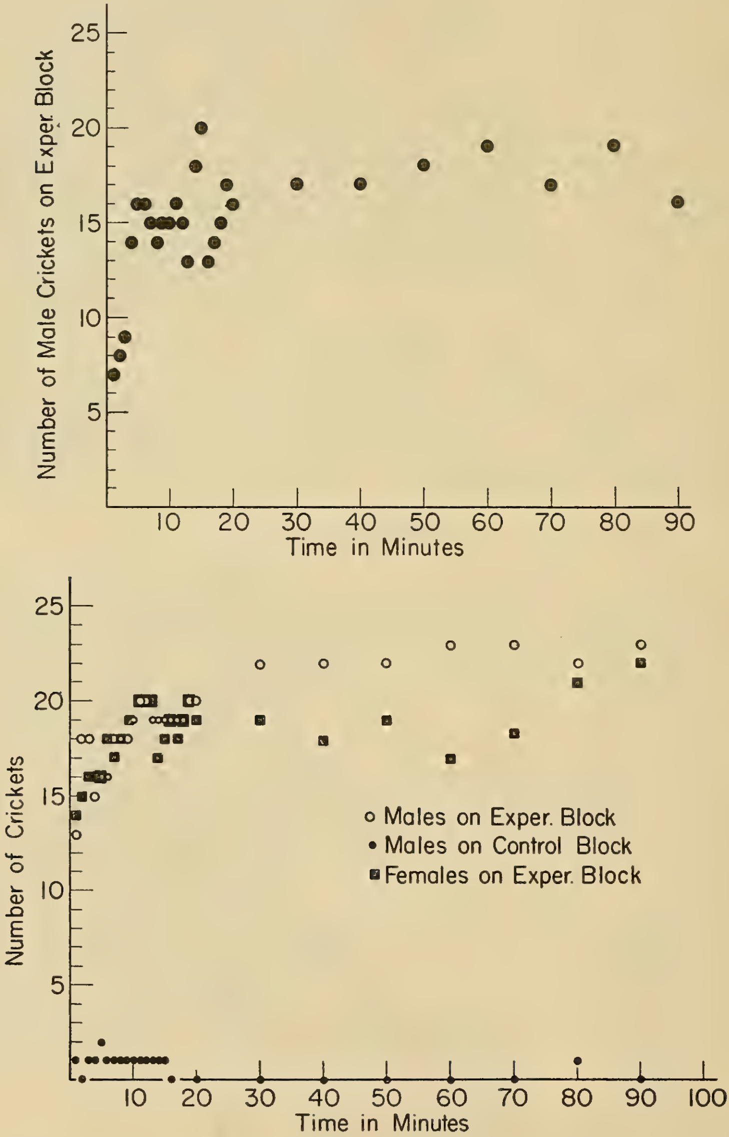

FIGURE 1a (upper). Occupancy of the uncovered experimental blocks by 24 isolated males. 1b, (lower) The same for 12 social groups, each consisting of 2 males and 2 females.

was perceived through the paper, one would expect most crickets on the paper-covered experimental blocks. If the cues were tactile, visual or a chemical which could not be perceived through the paper, one would expect the crickets to be evenly distributed between the two blocks. Readings of the position of the crickets were made at least two hours apart, and block position was not randomized after each reading.

The results were completely unexpected, for most males were on the paper-covered controls (Fig. 2). There were 70 observations of males on control blocks but only 16 on the experimentals for the first five observation periods. Corresponding values for females were 36 and 32, suggesting that females were distributed randomly between the block types. The data strongly support the hypotheses that: (1) the factor responsible for aggregation is not perceived through the paper; (2) a factor responsible for inhibiting male aggregation (but not female) on the conditioned blocks is perceived through the paper. In the second case, a mobile chemical is suggested as the dispersing agent.

These unexpected findings led us to believe that some uncontrolled artifact was operating, so the series was continued with one modification. The tissue covering was removed from both blocks within three randomly selected cages; the blocks in the other two remained unchanged. During the subsequent five periods there were 39 observations of males on the uncovered experimental blocks and 11 on the controls. For females, the comparable figures are 29 and 14. Hence, the attracting factor had been present all along during the first 5 readings, and during the second 5 readings both males and females responded to it as expected originally.

The animals in the other two cages were distributed randomly between the paper-covered blocks, with 23 observations of males and 17 of females on the control blocks and 17 of males and 13 of females on the experimentals. This randomization is interpreted as indicating that the dispersant was no longer effective, perhaps due to loss through gaseous diffusion or bacterial decay, and the attractant still was not perceivable through the paper. The dispersant against males appears to be effective up to 30 hours (Fig. 2a).

Series 4

These experiments were designed to re-test series 3 and to see which sex produced the dispersal pheromone. The experimental blocks were conditioned in cages containing only males (4a) or females (4b). For the 4a tests, there were four cages containing four males and another four with four females. Randomization of blocks between the two sites occurred after each reading, but otherwise the procedure was the same as in series 3.

In 4a (Fig. 3) there were 46 observations of males on the paper-covered control blocks and 12 on the male-exposed experimentals for the first five readings ($\chi^2 = 19.8$, D.F. = 1, $P_{0.995} = 7.88$) and 39 and 18 records of females on control and experimental blocks, respectively ($\chi^2_c = 7.0$, D.F. = 1; $P_{0.99} = 6.63$). Hence, the male data corroborate series 3 and show further that males both produce and respond to some dispersant on the experimental blocks. The females also appear to respond negatively to the male-conditioned blocks.

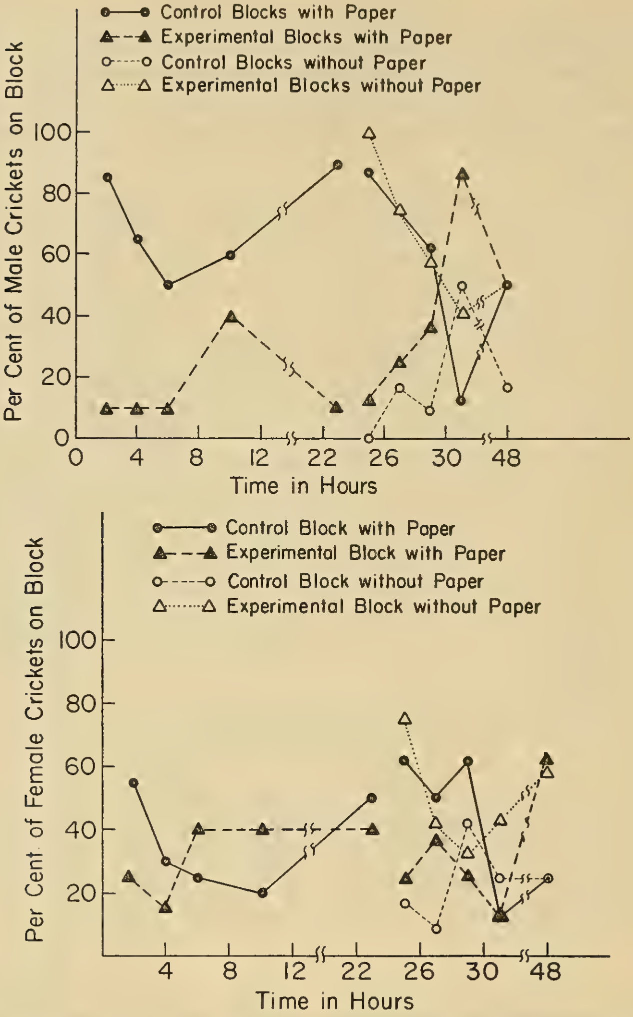

FIGURE 2. Occupancy of paper-covered control and experimental blocks, the latter conditioned in community cages. a (upper). Occupancy by males. b (lower). Occupancy by females.

Twelve hours after the start of 4a the paper was removed from the experimental and control blocks in two randomly selected cages containing males. The subsequent readings were: 6 observations of males on paper-covered experimental blocks and 26 on covered controls ($\chi^2_c = 11.2$, D.F. $= 1$; $P_{0.995} = 7.88$); 29 observations of males on exposed experimental blocks and 9 on corresponding controls ($\chi^2_c = 9.50$; D.F. $= 1$; $P_{0.995} = 7.88$). Hence, the effectiveness of both the dispersant and attractant remained high throughout this entire test.

The paper was also removed from two of the cages with females and the subsequent observations were: 24 of females on covered controls and 12 on covered experimental blocks ($\chi^2_c = 3.2$; D.F. $= 1$; $P_{0.95} = 3.84$); 1 of females on uncovered controls and 35 on the corresponding experimental blocks. These data and the preceding show that both sexes respond positively towards a male-produced attractant.

Series 4b duplicated 4a except that the blocks were conditioned in all-female cages. There were four test cages with four females in each and a similar arrangement with males. There was no evidence to show that the females produced a dispersant (Fig. 4). During the first five readings there were 21 records of males on the covered experimental blocks and 28 on the controls ($\chi^2_c = 0.72$; D.F. = 1; $P_{0.95} = 3.84$); in cages of females there were 24 and 23 cases, respectively.

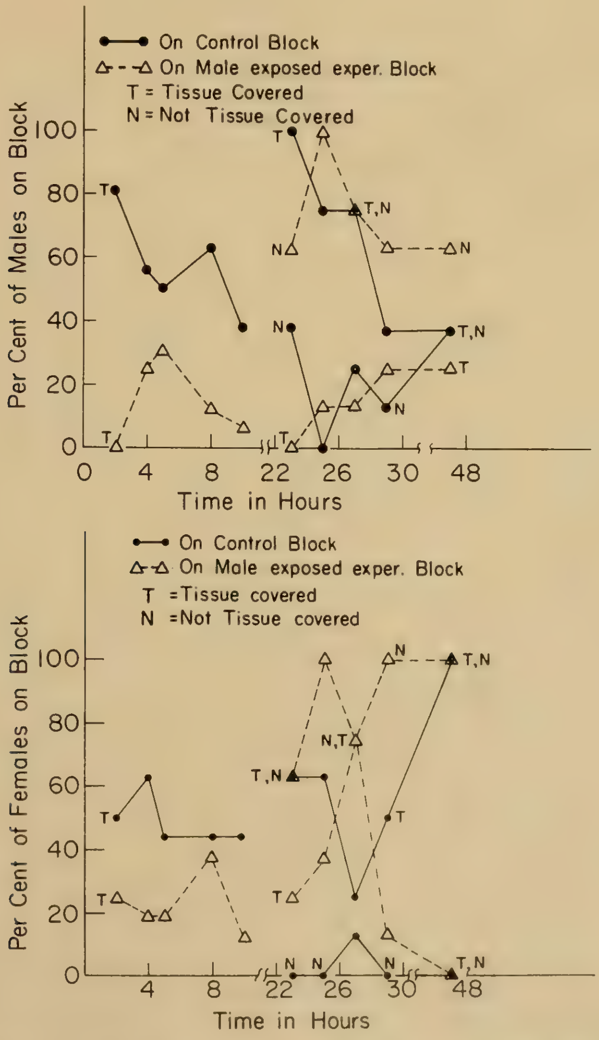

FIGURE 3. Occupancy of paper-covered control and experimental blocks, the latter conditioned by males. a (upper). Occupancy by males. b (lower). Occupancy by females.

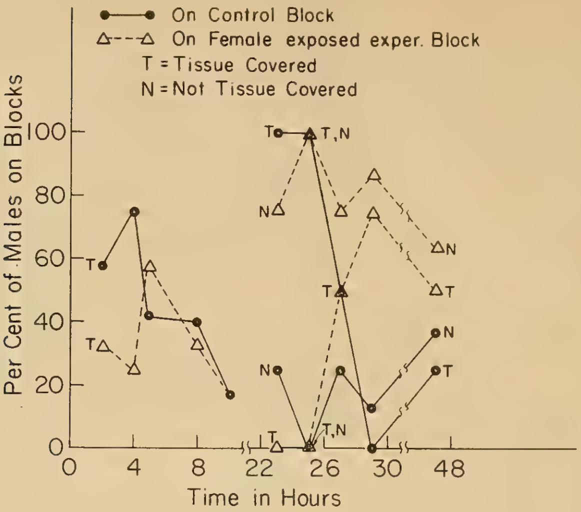

FIGURE 4. Occupancy of paper-covered control and experimental blocks, the latter conditioned by females.

The paper was removed from two of the three cages with males and from two of the four with females. For the males in the single cage with paper-covered blocks, there were 7 records of males on the experimental blocks and 11 on the controls. These extremely limited data suggest that no dispersant was present and that the males were distributed randomly. The males in the other terraria aggregated strongly; 32 observations for the uncovered experimental blocks *versus* 8 for the controls $\chi^2_c = 13.2$; D.F. = 1; $P_{0.995} = 7.88$).

Series 5

The final series was a complete re-run of series 4 with the blocks being conditioned under more controlled circumstances. Four 16-cm. blocks were placed in a terrarium containing 100 males or females and were left there for 6 days. Then the exposed blocks and the controls were covered with two layers of 18 cm. diameter filter paper. The tests were run in 16 five-gallon terraria, eight with male-conditioned experimental blocks (5a) and eight with female-exposed ones (5b). Six males or six females were introduced into half of the test cages of each category. The position of the two blocks was randomized after each reading.

The results for the males in 5a fully corroborated previous findings for the first 27 hours with 101 records of males on covered controls, 48 on the experimentals ($\chi^2 = 702$) and 19 on the cage floor (Fig. 5a). Furthermore, the effect of the treatment remained relatively constant for the first 27 to 30 hours of the experiment. After 27 hours of testing the paper was removed from both blocks in two randomly selected cages and unchanged in the other. The crickets in the former cages now switched strongly to the male-exposed blocks (31 on the exposed to 3 on controls) while those in the latter seemed to fluctuate randomly between the two blocks (14 on the paper covered controls and 18 on the corresponding experimentals).

The experiments testing females on male-exposed blocks again showed that females can respond negatively to some factor produced by males. During the first 10 hours of testing the females avoided the paper-covered male-exposed blocks, but, unlike the previous case with males, the response to the dispersant declined continuously (Fig. 5b) and at some period between 10 and 23 hours,

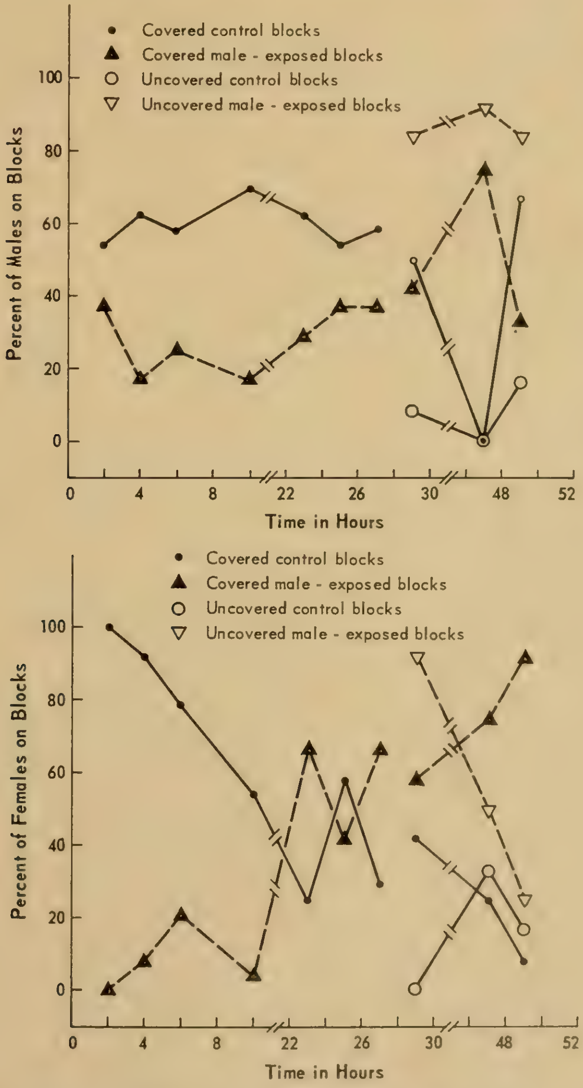

FIGURE 5. Occupancy of paper-covered control and experimental blocks. The experimental blocks were conditioned by placing four in a terrarium housing 100 males for six days. a (upper). Occupancy by males. b (lower). Occupancy by females.

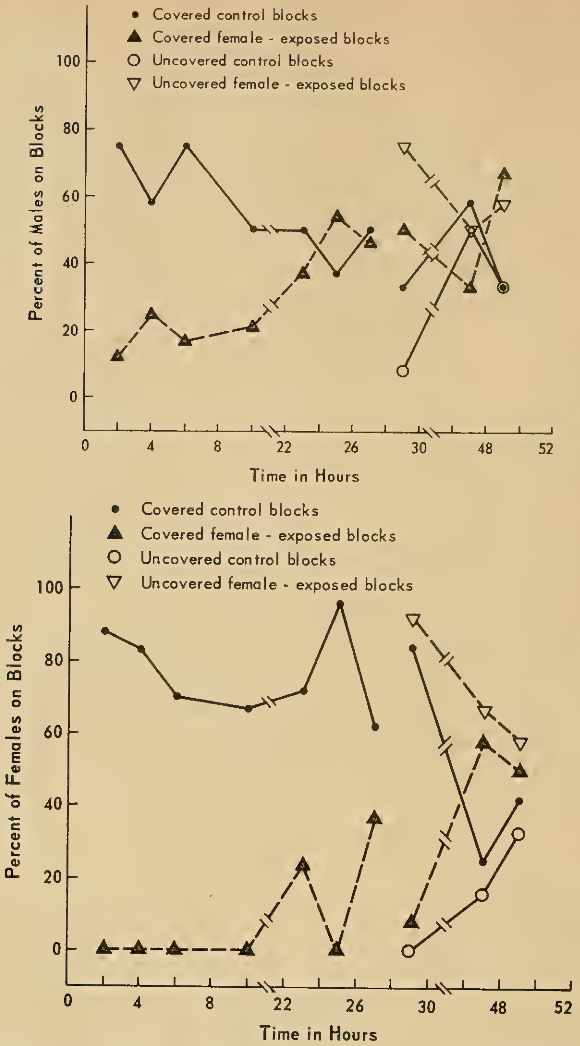

FIGURE 6. Occupancy of paper-covered control and experimental blocks. The experimental blocks were conditioned by placing four in a terrarium housing 100 females for six days. a (upper). Occupancy by males. b (lower). Occupancy by females.

the females began to respond randomly to the two blocks. This response is interpreted as indicating that the females respond less strongly to male-produced dispersant than do males, but that if it is strong enough, they will avoid blocks saturated with the dispersant. After 27 hours, the females in the cages with papered blocks began to shift over to the covered experimentals (21 on experimentals to 9 for controls), suggesting that the attractant was finally perceived through the paper after the dispersant had been dissipated. Females again preferred the uncovered experimentals to controls (20 to 6).

In series 5b, female-exposed blocks were used. Unlike earlier cases, the males initially reacted negatively to the female-exposed, covered experimental blocks, but this response decreased so that somewhere between 10 and 23 hours after the start, the males were responding randomly to the two blocks (Fig. 6a). This behavior remained constant for the rest of the experiment using paper-covered blocks. Most males in those cages with uncovered blocks were on the experimental blocks rather than controls (22 to 11; $\chi^2_c = 3.0$, D.F. $= 1$, $P_{0.95} = 3.84$), but the difference was not significant.

The final test was of females on female-exposed blocks and like the analogous case for males, there was a relatively constant avoidance of paper-covered female-exposed blocks for at least 29 hours (Fig. 6b). For the first 7 readings, there were 129 observations of females on controls and only 15 on experimentals. The cases with uncovered blocks again showed females selecting the exposed blocks, as expected.

DISCUSSION

A recent review of insect pheromones (Butler, 1967) indicated that most identified to date operate as alerting substances, trial markers, sex attractants, aphrodisiacs, aggregating substances and as controllers of development, but that few function in promoting dispersal. However, Naylor (1959) did show that a chemical dispersant operates under certain conditions in populations of the flour beetle, *Tribolium confusum*. The existence of pheromonal dispersants among vertebrates has often been suggested on the basis of morphological and descriptive behavioral studies, with the urination of canids upon conspicuous structures often cited as an example. Marler and Hamilton (1966) have briefly reviewed the subject, but the experimental evidence for the function of such urinary factors as territorial markers or dispersants is scanty.

Crickets are known to be territorial, and the role of the song in aggregation and dispersal has been worked out (Alexander, 1961). The evidence in this paper shows that cues other than auditory also operate in regulating the local distribution of one species of cricket. One set of factors, produced as crickets occupy some substrate, serves to attract other crickets to that conditioned substrate. Preliminary experiments, not reported here, suggest that the attracting factor is probably composite in nature, and since conditioned blocks covered with paper fail to attract crickets, it is clear that visual, tactile and non-mobile chemical cues are all possible contributors to the attractant. Norris (1963) believed that ovipositing female desert locusts deposited a chemical in the sand which enhanced the desirability of that area for other ovipositing females. In *A. domestica* both sexes produce an attracting factor(s), and both respond to it.

The action of the dispersant is seen best when the conditioned blocks are covered with two layers of filter paper so that the effectiveness of visual, tactile and non-mobile chemical cues is eliminated or at least reduced. The most likely nature for the dispersant is a somewhat mobile compound. While this compound may not be a pheromone using a strict definition, it has certain of the characteristics of pheromones (Karlson and Luschar, 1959). First, it is produced, at least indirectly, by the crickets, and is apparently a chemical. Second, it affects other conspecific individuals. Third, it produces a definite behavioral reaction, namely dispersion under conditions of the experiment.

Both sexes can produce the dispersant, and both can respond to it. However, experiments of series 3, 4 and 5 show that the two statements above are misleading unless the time factor is considered, and this discrepancy will be explained later. It is clear that both sexes respond more negatively to the dispersant produced by their own sex than to that of the opposite sex, suggesting several alternatives, namely that each sex: (1) produces a different dispersant; (2) produces different amounts of the same dispersant; (3) responds differently to the same dispersant; (4) some combination of possibilities 1 to 3. We cannot distinguish between these possibilities at this time.

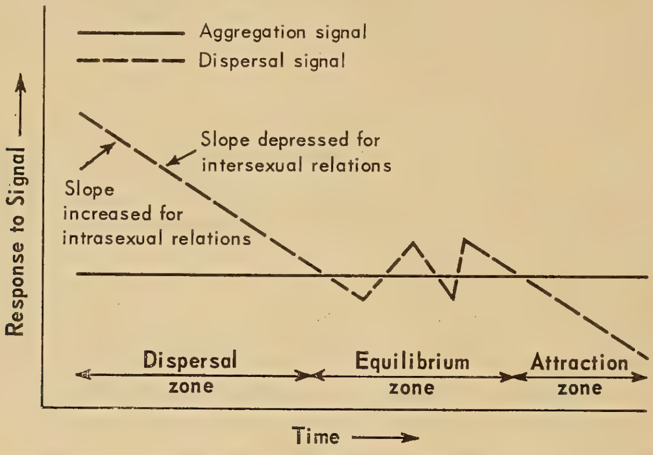

FIGURE 7. Model of the interaction of the dispersant and attractant in influencing the local distribution of the European house cricket, *Acheta domestica.*

A schematic representation of the action of this antagonistic system (attractant *versus* dispersant) has been devised which describes the experimental results obtained when the covered blocks are used (Fig. 7). The attractant, regardless of its physical nature, lasts much longer than does the dispersant, which disappears (through destruction or diffusion) within about 24–30 hours. The response of crickets of both sexes to the attractant is represented by a horizontal line and that to the dispersant by a line descending to the right. The area to the left of the intersection of these two lines is termed the dispersal zone, for here the effect of the dispersant overrides that of the attractant although both are present. The slope between the two lines is steeper when one considers the reaction of either sex to the dispersant produced by that sex. The early observations summarized in Figures 2a, 3, 5 and 6, represent this situation; corresponding stages in Figures 2b and 4 do not.

At the intersection of the two lines the two sets of antagonistic factors are in balance and the crickets will respond randomly to them as long as they remain in this condition. This part of the graph is termed the equilibrium zone and is represented by the first five observation periods in Figures 2b and 4 but by later stages in the other figures (*e.g.*, observations 6 through 10 in Figure 6a for covered experimental and control blocks). Presumably, the lack of a disperant zone in Figures 2b and 4 was caused by an insufficiency of dispersant being deposited on the experimental blocks before the start of the experiment so that the crickets reacted randomly towards the blocks.

The final zone, the attraction zone, begins when the dispersant becomes even more depleted, and the positive movement of the crickets towards the attractants becomes ascendent. This stage is well represented by observations 8 through 10 in Figure 5b. In many other cases, the experiments did not go long enough to enter this stage (*e.g.*, Fig. 6a).

On the basis of the proposed scheme, one would suspect that the attractant-dispersant system might operate as follows: The two factors would be deposited at any site (a burrow, call perch, etc.) which was occupied by a cricket. The longer the cricket remained, the more attractant-dispersant would be deposited. In turn, this place would be sought by other crickets for several reasons: (a) because of the structural and environmental factors which attracted the first cricket; (b) because of social interactions; (c) because of the presence of the attractant. It is to the resident cricket's advantage, however, to reduce aggregation, particularly unisexual groupings, to a level which the resident can control. The territorial system, with auditory, visual and pheromonal components, would act to reduce aggregation to that level. An immediate advantage of the attractant-dispersant system over the auditory system to a resident would be that the dispersant would be active at times when the resident had temporarily left the conditioned site. A long-term advantage of the system to the species would be that vacated favorable sites would not be stigmatized for long periods since the attractant would soon take precedence over the disappearing dispersant.

It is a pleasure to acknowledge the financial support of our fellow citizens for this research through a National Science Foundation Science Faculty Fellowship to Sexton and an NSF research grant (GB-3063). One of the experiments was run by graduate students enrolled in Hess' class. Discussions with Gordon Burghardt, Erich Klinghammer, Ben Beck and Thomas Uzzell were extremely useful in clarifying our thinking.

Summary

1. The European house cricket, *Acheta domestica,* will condition blocks of wood by depositing unknown classes of compounds upon them.

2. Crickets will occupy conditioned blocks in preference to unconditioned ones. The attractants on the conditioned blocks are produced by both sexes and both sexes respond positively to them.

3. When conditioned and control blocks are covered with tissue or filter paper, the crickets will now avoid the conditioned blocks, indicating the presence of a dispersant which was masked in tests carried out without a covering (as in [2] above).

4. Both sexes produce and both avoid the dispersant. Each sex responds more negatively to the dispersant of its own sex than to that of the opposite.

5. The dispersant appears to be a volatile compound which is ineffective after about 24 hours. The attractant is much more stable.

6. A model for the interaction of the attractant-dispersant is presented.

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OCCURRENCE OF PELAGIC LARVAE IN SPIO SETOSA VERRILL, 1873 (POLYCHAETA: SPIONIDAE)[1]

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Reproduction and larval development in *Spio setosa* Verrill, 1873, following the spring spawning period has been reported (Simon, 1967). Then, I showed that females spawned within a mucus tube enclosed by the adult sand tube and exhibited brood protection by remaining with the developing young. All development occurred within the adult tube and no pelagic stage was found. It was suggested that *S. setosa* had an additional spawning period in the fall resulting in pelagic larvae. This paper confirms the existence of a fall spawning period, describes the pelagic larvae for the first time, and discusses the significance of two spawning periods and two developmental patterns in *Spio*.

MATERIALS AND METHODS

Plankton was collected in approximately biweekly surface samples taken with Number 20 and Number 9 mesh nets in Great Harbor, Woods Hole, Massachusetts, throughout 1965 and 1966. Polychaete larvae were removed from these samples and cultured in the laboratory through metamorphosis and juvenile stages in small Stender dishes containing 15 ml. of autoclaved sea water held at a constant temperature of 10° C. Water was changed and cultures were fed 1–2 ml. of a dense, bacteria-free culture of the algae *Phaeodactylum* and/or *Isochrysis* weekly. When metamorphosis was imminent, 1–2 cc. of air-dried, autoclaved, sandy-mud substratum from Buzzard's Bay (off Nashawena Is.) was introduced into the cultures. Animals lived, metamorphosed, and grew rapidly under these conditions. Larvae reared through young bottom stages in the laboratory conformed in morphology and size with freshly collected animals. At this writing, three individuals are still in culture after a period of more than 18 months.

RESULTS

Occurrence of pelagic larvae

Larvae of *Spio setosa* were present in the Woods Hole plankton from mid-October to mid-February during 1965 and 1966. Pelagic developmental stages from 4-setigers to 22-setigers were found. Other spionid larvae present during this period were: *Spiophanes bombyx* (Claparède, 1870); *Polydora caulleryi* Mesnil,

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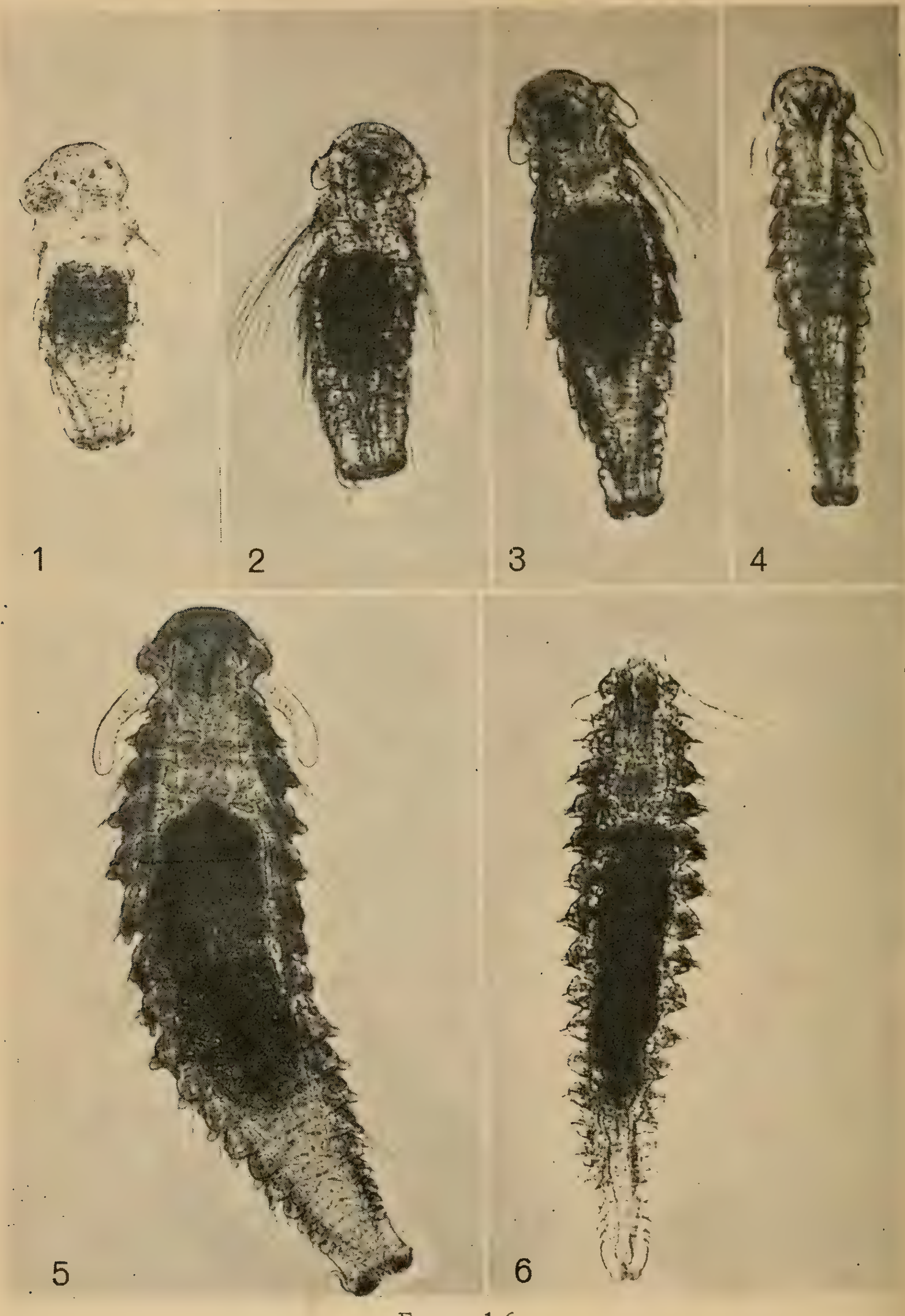

Figures 1–6.

TABLE I

A comparison of characteristics of benthic and pelagic larvae of S. setosa

	Benthic larvae	Pelagic larvae
Period of occurrence	March–June	October–February
Size	larger	smaller
Location of gastrotrochs	setigers 3, 5, 7, 9, 12, rarely 14.	setigers 3, 5, 7, 9, 12, 14, rarely 15
Larval notosetae	uniserrate	uniserrate & multiserrate
Larval neurosetae	uniserrate; appear at 7-setigers	uniserrate; appear at 10- to 12-setigers
Adult neuropodial hooded crochets	appear in setiger 6 and posteriorly at the 9-setiger stage	appear in setiger 9 and posteriorly at the 11- to 12-setiger stage
Pigmentation	usually lacking	paired lateral black dorsal spots from setiger 2 posteriorly; black spots at base of all dorsal cirri;brown spot at base of palps, black bar on ventral lips
	gut black at metamorphosis	gut black at all stages
Anal cirri	appear at 11-setigers	appear at 13- to 16-setigers
Feeding	adelphophagia until 7-setigers; adult mode thereafter	planktotrophic from 6-setigers to metamorphosis; cannibalism (?)
Metamorphosis	at 15- to 17-setigers	at 18- to 20-setigers

1897; *Polydora commensalis* Andrews, 1891; and *Scolecolepides viridis* (Verrill, 1873). Although quantitative samples were not taken, it was clear that *Spio* larvae were the most abundant and dominant polychaete larvae in the plankton during November and December of both years.

Morphology of pelagic larvae of Spio setosa

Pelagic larvae of *Spio* taken in the fall agreed in most respects with benthic larvae observed in the spring (see Simon, 1967; Table I; and Figs. 1–6). Only differences observed between normal spring benthic and fall pelagic larvae will be noted here.

Although the form of pelagic larvae was identical to that of benthic larvae, pelagic larvae of 6- to 13-setigers were smaller than benthic larvae of the same stages (Fig. 7).

FIGURE 1. Pelagic 6-setiger larva of *S. setosa,* dorsal view. Photomicrograph of living specimen. Actual length = 0.579 mm.

FIGURE 2. Pelagic 9-setiger larva of *S. setosa,* dorsal view. Photomicrograph of living specimen. Actual length = 0.739 mm.

FIGURE 3. Pelagic 12-setiger larva of *S. setosa,* dorsal view. Photomicrograph of living specimen. Actual length = 0.998 mm.

FIGURE 4. Pelagic 13-setiger larva of *S. setosa,* ventral view. Photomicrograph of living specimen. Actual length = 0.998 mm.

FIGURE 5. Pelagic 19-setiger larva of *S. setosa,* dorsal view. Photomicrograph of living specimen. Actual length = 1.846 mm.

FIGURE 6. Metamorphosed 19-setiger larva of *S. setosa,* ventral view. Photomicrograph of living specimen. Actual length = 2.352 mm.

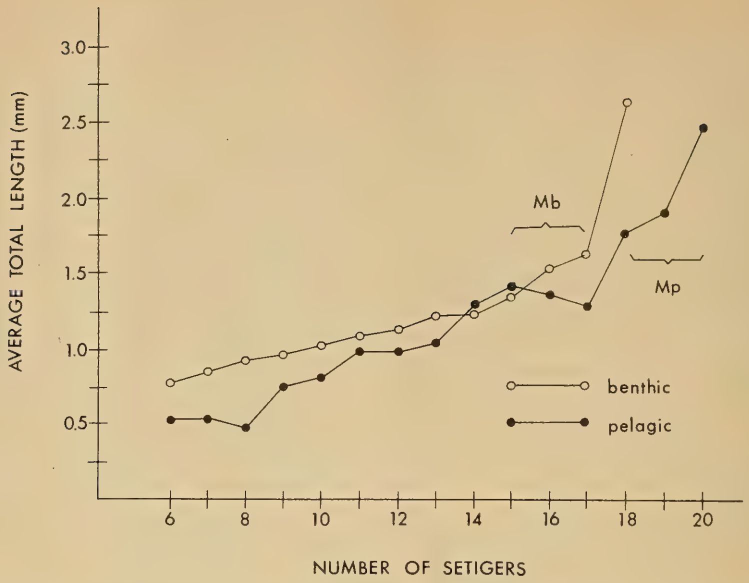

FIGURE 7. A comparison of the size of pelagic and benthic larvae of *S. setosa* at various stages. Mb = stage at metamorphosis of benthic larvae; Mp = stage at metamorphosis of pelagic larvae. Data for benthic larvae taken from Simon (1967). Averages for pelagic larvae based on measurements of at least 8 specimens.

Ciliation patterns varied only slightly between larval types. Both possessed a prototroch, telotroch, and nototrochs on all segments posterior to segment 3, as well as a neurotroch and ciliated pit on segment 2. The arrangement of gastrotrochs differed: older benthic larvae possessed gastrotrochs on segments 3, 5, 7, 9, and 12, and, rarely, on segment 14; pelagic larvae consistently possessed gastrotrochs on segments 3, 5, 7, 9, 12, 14, and occasionally on segment 15.

The types and arrangement of setae differed somewhat in the two larval types. Both had uniserrate larval notosetae at all stages, and these were shed at metamorphosis. In pelagic larvae, however, one or two multiserrate larval notosetae were rarely encountered in the first or second setiger. In benthic larvae, uniserrate larval neurosetae appeared at the 7-segment stage. These neurosetae were found later in planktonic larvae (10- to 12-setigers). Adult, bidentate, hooded crochets appeared in benthic larvae by the 9-segment stage in all segments posterior to setiger 5. In pelagic larvae, the same type of crochets appeared at the 11- to 12-setiger stage, and were found in all segments from setiger 9 posteriorly. After metamorphosis, crochets were gradually lost from the first 13 segments in both larval types. Adult limbate capillaries appeared in the neuropodia and notopodia accompanying the larval setae in both larval types, so that by metamorphosis adult setae were well represented.

An additional distinction between benthic and pelagic larvae exists in pigmentation patterns. Whereas benthic larvae were essentially unpigmented, or only rarely possessed black chromatophores, pelagic larvae always had a rigid pattern of pigment spots. These consisted of paired, lateral, small black spots on the dorsum of all segments from setiger 2, posteriorly; similar black chromatophores at the bases of all dorsal cirri; a dark brown spot at the base of each developing palp; a ventral black bar on the lateral lips; and paired black chromatophores at the lateral sides of the pygidium (Fig. 8). The gut of pelagic larvae could be clearly distinguished by the presence of gray-green to black pigment in all stages, whereas benthic larvae developed this pigment only just prior to metamorphosis. A yellowish-brown prostomium and pygidium were characteristic of both larval types.

The development of anal cirri occurred later in fall than in spring larvae. While anlagen of two pairs of anal cirri appeared at the 11-setiger stage in spring larvae, they did not appear until the 13- to 16-setiger stage in fall larvae. In both larval types the dorsal anal cirri were colorless, narrow, and finger-like while the ventral anal cirri, at least until after metamorphosis, were yellowish, rounded, and possessed a number of gland cells.

The development of branchiae occurred at the same stage in both spring and fall larvae. The first branchial anlagen to appear were those on setigers 3 through

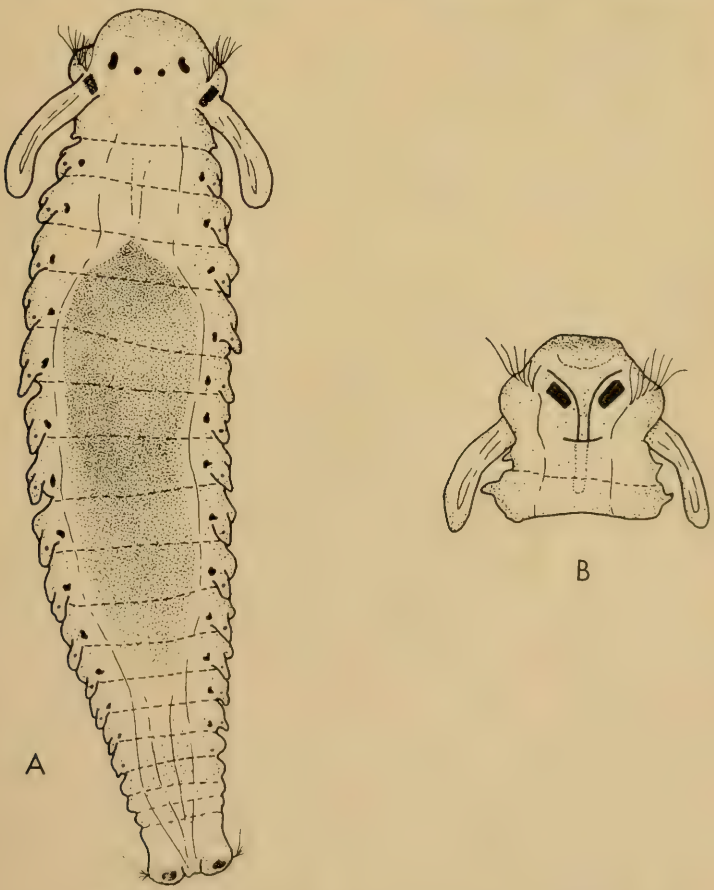

FIGURE 8. Pelagic 19-setiger larva showing pigment pattern. A. Dorsal view. B. Ventral view of head. Drawn from photomicrographs of living specimens. Setae omitted.

11. As fall larvae metamorphosed at a slightly later stage than spring larvae (see below), planktonic forms were found with branchial anlagen, while benthic larvae never possessed them until after metamorphosis. Addition of branchiae on the anterior two segments occurred after metamorphosis in both cases, appearing on segment 2 by the 30-setiger stage and on the first segment by 40-setigers.

Benthic larvae were found to exhibit adelphophagia until the 7-segment stage, subsisting on extrinsic yolk material present within the egg mass. From 7-segments until metamorphosis, they fed on detritus and benthic diatoms brought into the tube by the respiratory currents of the female parent (Simon, 1967). Examination of gut contents of recently collected larvae suggested that pelagic forms fed on planktonic diatoms. In culture, larvae, juveniles, and adults were kept for over one year on a diet of only *Phaeodactylum.* Cannibalism was observed in freshly obtained plankton samples and in laboratory culture where other food was abundant. Phytoplankton feeding appeared to occur by the action of the oral cilia. Only in the case of ingestion of large particles (polychaete larvae, for example) were muscular distention of the mouth and swallowing movements observed. In culture, food was always provided in excess and much of the ingested phytoplankton passed through the gut apparently undigested. This material was compacted into fecal pellets containing digested phytoplankton tests, living phytoplankters, bacteria, and mucus. In fact, larvae, juveniles and adults could subsist for periods longer than one month with a single initial addition of phytoplankton, fecal pellets being ingested and reingested. Juvenile animals even utilized fecal pellets as raw-materials for tube formation, compacting them with mucus, when no other materials were present.

Metamorphosis and the effects of lack of substratum

Metamorphosis of pelagic larvae occurred at a later stage than that of benthic larvae. While spring forms metamorphosed at the 15- to 17-setiger stage, fall larvae normally delayed until 18- to 20-setigers. However, unmetamorphosed larvae were observed in the plankton possessing up to 22-segments, and metamorphosed young were seen as small as 14-segments, indicating considerable variation. At metamorphosis, pelagic larvae sank to the bottom, burrowed into the substratum, and took up a benthic existence. Larval setae and the ciliary bands of the prototroch, telotroch, neurotroch, ciliated pit, and the gastrotroch were shed. Nototrochal bands were retained on the segments bearing branchiae. In laboratory cultures larvae provided with Buzzard's Bay sediment which had been air-dried and autoclaved always metamorphosed at 18- to 20-segments.

The effects of lack of substratum on metamorphosis of pelagic larvae were tested in the laboratory. Larvae reared in culture vessels without substratum showed three responses. First, the majority of larvae metamorphosed, forming mucus tubes on the bottom of the culture vessel, lack of substratum having no effect on metamorphosis. Secondly, some individuals remained unmetamorphosed and continued to increase in size and number of segments, growing to 22 segments by two months after normal metamorphosis was expected. Thirdly, some specimens retained larval characteristics and increased in size without adding new segments. One 14-segment larva was maintained in culture for two months, increasing from the normal length for a larva of this number of segments of 0.9

mm. to the giant size of 1.5 mm. Thus, absence of substratum affected metamorphosis of at least a portion of the pelagic larval population. A similar delaying of metamorphosis has been shown with benthic larvae (Simon, 1967). The critical factors associated with lack of substratum have not been investigated.

Size, growth, and effects of salinity

Figure 7 records the size of fall pelagic larvae at each stage of development compared with that of benthic larvae. The fact that fall larvae of 6- to 13-segments were smaller than comparable spring larvae might be expected as the former were pelagic and planctotrophic while the latter were benthic and exhibited adelphophagia. After attaining about 13-segments, both larval types attained the same sizes.

The time sequence of natural pelagic development has not been determined. However, by extrapolation from multiple observations of many larvae held in culture conditions, about one month was required to grow from 6- setigers to metamorphosis (20-segments). As surface sea water temperatures in nature during the period from October through February (as measured in the MBL sea water system) dropped from about 15° C. to 2° C., and phytoplankton concentrations usually decrease to low levels during this period (Lillick, 1937; Fish, 1925) a longer time would be expected to elapse prior to metamorphosis in natural populations. Benthic larvae cultured at temperatures of 8° C to 11° C. also required about one month to reach metamorphosis (Simon, 1967).

The effects of various salinities on the development of pelagic larvae were determined as follows: Six larvae were placed in each of three salinities: 100% (32‰); 75% (about 24‰); and 50% (about 16‰). Larvae, observed and measured periodically for six months, lived, grew well, and metamorphosed in all salinities. Initially larvae appeared opaque, swollen, and were inactive in 50% sea water. After three weeks, however, signs of swelling disappeared and all larvae were active and of normal appearance. A morphological anomaly appeared in larvae held in 50% and 75% sea water after approximately 4 weeks. In addition to normal gastrotrochs on setigers 3, 5, 7, 9, 12, and 14, larvae in dilute sea water possessed additional incomplete bands on setigers 4, 6, 10, 13, and 15 through 19. The additional gastrotrochs were composed of one or two medial ciliated cells rather than a complete row of cells across the ventral surface of the larva. The significance of the added cilia is not clear. Reduced salinity did not affect size, number of segments, or rate of growth. As adult *Spio* populations were found in areas of reduced salinity, one would expect that both larvae and adults could tolerate lowered salinities without adverse effects.

Swarming of juveniles

Night plankton tows made during late May and early June, 1966, yielded young metamorphosed specimens of *Spio setosa* from 6.0 to 30.0 mm. in total length, with a maximum of 96 segments. These animals conformed in size and number of segments with young *Spio* reared in laboratory culture from larvae collected in the fall and winter plankton. It was impossible to culture swarming juveniles *in vitro* for more than a few hours. Juveniles of other benthic polychaetes were also found in the plankton during the same period [*Arenicola* "Benham-stage"; *Scalibregma* *inflatum* Rathke, 1843; and *Pherusa affinis* (Leidy, 1855)]. The nature of the swarming of juveniles and reasons underlying this behavior are unknown (see discussion).

DISCUSSION

A number of invertebrates in different groups are known to exhibit two (or more) developmental patterns, either at different times of the year or in different areas within their geographic ranges. This phenomenon, termed poecilogony by Giard (1904), was reviewed by Thorson (1950) who concluded that poecilogony was of general importance only in the Polychaeta.

The differences in developmental patterns in polychaetes involve: (1) the length of pelagic life; and (2) planctotrophic or lecithotrophic larval nutrition. Within the same species in one part of its range, or at a given time of year, extended planctotrophic pelagic larvae occur, while in another region or at a different time of the year, no pelagic larvae occur and the young subsist on intrinsic or extrinsic yolk material (adelphophagia). The following polychaete species have been stated to exhibit poecilogony: *Spio martinensis* Mesnil, 1896 (Mesnil, 1896; 1897; 1916; 1925; Mesnil & Caullery, 1917; Caullery & Mesnil, 1918; Hannerz, 1956); *Pygospio elegans* Claparède, 1863 (Söderström, 1920; Smidt, 1951; Hannerz, 1956); *Nereis pelagica* Linné, 1761 (Herpin, 1925; Wilson, 1932); *Nereis zonata* Malmgren, 1867 (Thorson, 1950). In other instances, where a single species was thought to have two decidely different developmental patterns, two separate species have been shown to be involved. For example, Guberlet (1934) reported a species listed as *Arenicola claparède* Levinsen, 1883, to have non-pelagic development in Puget Sound, and Okuda (1938) recorded the same "species" to have pelagic larvae in Japan. Later, Healy and Wells (1959) and Wells (1963) showed that Guberlet's *A. claparède* should be referred to *Abarenicola claparède vagabunda* Healy and Wells, 1959, while Okuda's species should have been *Abarenicola pacifica* Healy and Wells, 1959. Similarly, *Platynereis dumerilii* (Audouin and Milne-Edwards, 1833) was reported to have pelagic ("planktogene") larvae and non-pelagic ("nereidogene") larvae (Hempelmann, 1911). It is now known that the non-pelagic ("nereidogene") larvae belong to a separate species, *Platynereis massiliensis* (Moquin-Tandon, 1860) (Hauenschild, 1951). To my knowledge, the best documented cases of poecilogony in polychaetes are those in the spionids *Spio martinensis*, *Pygospio elegans,* and now, *Spio setosa.* It is clear, however, that any "species" which exhibits more than one reproductive or developmental pattern should be examined critically to insure that two cryptic species are not involved. If it can be demonstrated that the same female of a given species spawns twice a year and the resulting eggs develop into different larval types, we can be assured that a single species is involved. On the other hand, if a single female can be shown to spawn only once a year, producing either only benthic or only pelagic larvae, then two separate, morphologically similar, species must be involved, as there is obvious reproductive isolation. I know of no cases of poecilogony in which a single female has been shown to produce both larval types. The difficulties in maintaining most polychaetes in laboratory culture from egg through reproducing adult are responsible for this lack of knowledge. Perhaps with the advent of recent culturing methods, rearing polychaete larvae to adulthood can be accomplished with the species involved. Until this is done, we must rely on the morphological taxonomic criteria set up by systematists, which so far indicate that *Spio martinensis, Spio setosa,* and *Pygospio elegans* are each but a single species.

The adaptive significance of poecilogony is clear. Once established in a given locale, benthic larvae (developing from the spring spawning in the case of *S. setosa*) allow large populations to be built up rapidly. Pelagic larvae (following the fall spawning in *S. setosa*) allow for dispersion by tides and currents, exposing individuals to new habitats. The plasticity of development allows the animal to take advantage of new habitats and, once established, to build up large populations.

The description of pelagic larval morphology presented here for *S. setosa* shows good agreement with that of benthic larvae described earlier (Simon, 1967) and with that of other pelagic spionid larvae (see Hannerz, 1956). The minor differences between pelagic and benthic larvae, mainly changes in the time of appearance of various structures, I consider to be of no taxonomic consequence. These differences probably reflect the different times involved in reaching metamorphosis in the two larval types. As stated earlier, no absolute time scale for development in natural larval populations is available. Laboratory cultures showed little difference in time for benthic and pelagic larvae to reach metamorphosis. This is most likely due to the fact that the two most important environmental parameters said to control rate of growth in nature, temperature and availability of food (Orton, 1920; Thorson, 1946, 1950), were held constant in the laboratory. Undoubtedly it would take naturally occurring planktonic larvae longer to reach metamorphosis than they did in the laboratory, and probably longer than that taken by naturally occurring benthic larvae due to the lower environmental temperatures and relative scarcity of food material in the fall (see above). Thorson (1950) stated that most planktotrophic larvae took one to three months to develop in the winter. As *Spio* larvae of one stage or another are present in the plankton for a four-month period (October to February), this time interval seems reasonable for naturally occurring *Spio* as well.

Mode of nutrition is one of the major differences between benthic and pelagic larvae of *S. setosa,* and probably accounts for the size differences observed. Benthic larvae were shown to exhibit adelphophagia up to the 7-segment stage, and then to take up the adult feeding type, utilizing organic detritus and benthic diatoms brought to them by the action of the ciliary currents of the female parent (Simon, 1967). Pelagic larvae, on the other hand, fed on phytoplankton and perhaps polychaete larvae of the same or different species. Other work on feeding in planktotrophic larvae has shown that, although a few forms utilize animal food and larger diatoms, the majority subsist on small phytoplankton organisms (Thorson, 1946, 1950). Culture observations on overfeeding in *Spio* larvae agree with what is known in most planktotrophic forms (Thorson, 1950). Similar observations on the ingestion and reingestion of fecal pellets were made by Johannes and Satomi (1966) in work on shrimp nutrition.

Although observations are few, initial phases of fall development (up to 6- or 7-setigers) appear to occur within the tube of the adult female of *Spio setosa* in much the same manner as in the spring. Egg masses were observed in the fall earlier (Simon, 1967) and pelagic larvae younger than 6- or 7-setigers were not found commonly in the plankton. Whether the young stages subsist on intrinsic yolk material or exhibit adelphophagia in the fall is unknown. In other species, the presence and degree of adelphophagia appear to be important in determining whether larvae will be benthic or planktonic and the duration of each larval type. In gastropods, Thorson (1935, 1950) suggested that embryos leave the egg capsules "when they have reached the stage of development which the nurse eggs at their disposal are able to support . . . it is evident that . . . 16 embryos in a single capsule will hatch at a much younger stage than a single embryo contained in a capsule with plenty of food at its disposal" (1950, p. 9). The number of nurse eggs, that is, the amount of extrinsic yolk material available for larval feeding, must control in part the type of larva, the stage at which larvae leave the egg mass and become planktotrophic, and thus the length of pelagic larval life. It would be helpful to know, in the case of *S. setosa,* the relative numbers of nurse eggs present in the spring and fall. If there were more nurse eggs available to each larva in the spring there would be little selective advantage to becoming pelagic in search of food. In fact, larvae distended with nurse eggs tend to be heavy and unable to swim. If there were fewer nurse eggs available to developing larvae in the fall, other sources of nutrition (*e.g.*, phytoplankton) would be necessary for survival. Unfortunately, no information as to the relative numbers of nurse eggs at different seasons is available for *Spio* or any other poecilogonic polychaete.

The effects seen in larvae of *Spio setosa* in response to reduced salinity might have been expected from a knowledge of the location of adult populations. As stated by Simon (1967), adult populations are usually found along the protected side of rock jetties, often associated with fresh-water streams emptying into small harbors and bays. Adult salinity tolerance have never been investigated. Lyster (1965) studied the effects of reduced salinity on larvae and adults of five polychaete species, and found that in most cases the tolerance to lowered salinities corre-
harbors and bays. Adult salinity tolerances have never been investigated. Lyster also noted a swelling of larvae, cessation of movement, and return to normal behavior after exposure to reduced salinity as that seen in *Spio* larvae. The significance of additional ventral ciliated cells found in response to salinity reduction is not known. It is tempting to postulate that lowering of buoyancy provided in 50% and 75% sea water (as opposed to 100% sea water) made additional bands "necessary" if the larva was to remain in the water column.

The effects of lack of substratum on metamorphosis in polychaetes were studied by Day and Wilson (1932, 1934) and Wilson (1937, 1948, 1950, 1952, 1953a, b). These studies suggested that polychaetes deprived of suitable sediment would not metamorphose or would delay metamorphosis for periods of several days to several months. Their findings were limited to *Ophelia, Notomastus,* and *Scolelepis.* The one species most studied in this regard, *Ophelia bicornis,* is a species which is known to have specific, rigidly defined habitat preferences (see Amoureux, 1960, 1962). The results obtained with *Spio* show quite a different picture, as the majority of larvae metamorphosed without substratum of any kind. Thorson (1966) indicated that although some species have specific substratum preferences and will not metamorphose without these conditions, most species are either not highly discriminatory at the time of normal metamorphosis, or become less discriminating the longer appropriate substratum is withheld. Likewise, some data from Muus (1966) would suggest that many polychaete larvae settle in great numbers in substrata unsuited to later life, and, in the course of a month or less, disappear completely from the bottom (see below).

The occurrence of juveniles of *Spio* in the plankton during the evenings of May, was reported by Moore (MS.). Other juvenile benthic polychaetes have also been reported swimming in the water column. The occurrence of the so-called "Benham-stage" of *Arenicola* was noted by Ashworth (1904), Blegvad (1923) and Thorson (1946). Thorson (1946) reported a number of "pelagic bottom stages" (metamorphosed young) of various echinoderms, prosobranchs, opisthobranchs, and other polychaetes (*Phyllodoce groendlandica, Harmothoë imbricata*). The explanation put forth was that these normally benthic forms found in the plankton represented "larvae metamorphosing in midwater and afterward continuing their pelagic life" (Thorson, 1946, p. 466). Day (1934) found that *Malacoceros fuliginosus* (= *Scolelepis*) metamorphosed while still swimming in laboratory cultures after prolonged periods without substratum. Berkeley and Berkeley (1963) encountered reproductive "neotenous" larvae of *Prionospio pinnata* Ehlers, 1901, and *P. ornata* Berkeley and Berkeley, 1961, off the Peruvian coast, postulating metamorphosis in midwater. Although this explanation might serve to explain the presence of pelagic juveniles of other species, it is not sufficient to explain the occurrence of *S. setosa* young in the plankton. The latest collection of pelagic larvae of *Spio* for 1966 was in mid-February, while juveniles were found in the night plankton for the first time in May. No *Spio,* larval or juvenile, were found in the plankton in March and April. Thus, larvae must have metamorphosed and burrowed into the bottom by the end of February, and in May gave up the benthic habitat for a pelagic one. Several explanations might account for this. First, and most likely, although the substratum appeared suitable for initiating metamorphosis and available food supply was sufficient for very young worms, juvenile forms of 3 to 4 months age might have found the habitat undesirable or food insufficient, thus swimming upward, allowing tides and currents to sweep them to new sites. This explanation finds some support in the findings of Muus (1966), who observed that a "great number of newly-settled bottom stages representing various animal groups occur at different seasons of the year (in the bottom), persist for a short period and then disappear almost completely . . . that vast numbers of planktonic larvae will settle on a substratum where they do not belong" (p. 290–291). A second explanation, although less likely, is that the number of larvae settling in a given area was great, and crowding of the growing young led to migration via the plankton.

It would seem of little adaptive advantage for benthic juvenile worms 6 to 30 mm. in length to expose themselves again to the rigors of the plankton and the added chances of predation. The fact remains, however, that it does happen.

SUMMARY

1. *Spio setosa* exhibits poecilogony, spawning once in the spring, resulting in benthic larvae; again in the fall, with pelagic larvae.

2. Pelagic larvae from 4- to 22-setigers were collected between mid-October and mid-February in Great Harbor, Woods Hole, Massachusetts. Larvae were reared in laboratory culture through metamorphosis and juvenile stages.

3. The morphology of pelagic larvae paralleled generally that of benthic larvae with the following exceptions: pelagic larvae from 6- to 13-setigers were smaller

than comparable benthic larvae; pelagic larvae had gastrotrochs on segments 3, 5, 7, 9, 12, 14, rarely on 15; a rigid pattern of black pigment spots was present on pelagic larvae; pelagic larvae were planktotrophic; metamorphosis occurred at 18- to 20-setigers.

4. In response to lack of substratum, most *Spio* larvae metamorphosed, forming tubes of mucus. Some larvae did not metamorphose for periods up to two months, either increasing in size and number of segments, or just increasing in size.

5. Larvae survived and metamorphosed in 50%, 75%, and 100% sea water. In dilute sea water, additional incomplete gastrotrochal bands appeared on segments 4, 6, 10, 13, and 15 through 19.

6. Juvenile worms 6 to 30 mm. long reappeared in the night plankton during May and early June.

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Note added in proof:

J. D. George (1967. J. Mar. Biol. Assoc., **47**: 75–79) has shown that both benthic and planktonic larvae may result from the fertilization of eggs from a single female of *Cirriformia tentaculata* (see Discussion above).

THE PHOTOTACTIC BEHAVIOR OF THE STARFISH, ASTERIAS AMURENSIS LÜTKEN

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Starfishes usually move into an illuminated area or toward a light source, but occasionally move away from it. Their behavior has been reviewed by Yoshida (1966). Some of the reports on tactic movements appear discordant and two opposite types of response, positive and negative, have been reported in the same species. Further, there are differences in opinion concerning the photoreceptive function of ocelli, which has been supported by some authors (Plessner, 1913; Just, 1927; van Weel, 1935; Diebschlag, 1938) but not by others (Mangold, 1909; Rockstein, 1956). The ray preference in locomotion which exists in some species (for review, see Reese, 1966) may complicate the issue. A difficulty may also exist in the variability in the behavior of starfishes under differing environmental conditions. This is so variable as to appear too complex for precise analysis and led Diebschlag (1938) to invoke the internal states of animals.

Starfishes move by coordinated stepping of tube feet and Smith (1945, 1950) has proposed that directional coordination is achieved by temporary dominance of one of five nerve centers in the circumoral nerve ring, though the number of the centers seems to be still a matter of debate (Kerkut, 1954). Since then, no work has been published specifically on the part played by such centers in connection with phototaxis.

Studying the action spectrum of reflex reactions led us to suggest that the ocellar and non-ocellar photoreceptors co-exist in the Japanese common starfish, *Asterias amurensis* (Yoshida and Ohtsuki, 1966). During the course of the study, we noticed that their locomotion was much affected by light. We therefore started to analyze the behavior of this species, attempting to elucidate the role of ocelli and the nervous system in determining the direction of locomotion.

MATERIAL AND METHODS

Individuals of *Asterias amurensis* Lütken of medium size with rays about 5–7 cm. long were used. They were kept in a laboratory tank with running sea water. In some cases a variety of operations was performed in order to study the role of ocelli and the nervous system. Ocelli were taken out, together with the terminal tentacle, by means of fine forceps. The radial nerve was transected near the proximal end and the oral nerve ring, in an interradius by means of a piece of razor. Sometimes an arm or arms were cut off either with the oral nerve ring left intact or a part of it attached. These operated specimens were kept in running sea water for at least four days for recovery, and completeness of the operation was checked on formalin-fixed specimens after use.

The experiments were performed in a dark room. Movements of the starfish were observed under a photographic safe light by placing them one at a time at the center of a black trough, 40 cm. wide, 60 cm. long and 10 cm. deep, containing sea water to a depth of 5–6 cm.

The direction of movement was expressed in one of the following three ways. *Method 1.* This was used when half of the trough was shaded. As is shown in Figure 1, the bottom of the trough was divided into 12 sections by white lines radiating from the center at intervals of 30°. The figure in each section may be called the directional index, the sign of minus indicating the shaded area. *Method 2.* This was used when an arm of stationary animals was stimulated by light or shadow. The stimulated arm was numbered I, the rest of the four arms being numbered respectively II, III, IV and V anticlockwise. The locomotory pattern was expressed by a circular histogram radiating from the central circle. The lengths of the heavily shaded bars show percentages of animals which started to move in the corresponding direction. Figures shown in the central circles indicate percentages of animals which did not move during the period of observation lasting for 1 minute. *Method 3.* This was used for experiments on moving animals. Here the leading arm was numbered I, the rest being numbered II–V as above. The locomotory pattern was expressed in a similar way as above, but the lengths of the bars and the figures in the central circles represent respectively percentages of animals which were moving in that direction and those which did not move at the time of observation, namely, 15, 35 and 55 seconds after stimulation.

In order to standardize the photo-physiological conditions, animals were either illuminated at 200 lux for more than 30 minutes or confined in darkness for more than 4 hours in running sea water. For standardization, it was also important to expose animals to air for 3–5 seconds. By this treatment, pre-existing tendencies to move with a certain ray in advance were abolished.

An ordinary electric bulb, 100V 40W, was used as a light source for illuminating a wide area. It was fixed 60 cm. above the center of the trough and the light intensity at the bottom was about 200 lux. For illuminating a restricted area, a microscope lamp, 6V 30W, was fixed at the end of a horizontally flexible arm 50 cm. above the trough. The stray light was reduced by using a cylindrical opaque shield, 6 cm. in diameter and 44 cm. long. The light intensity at the bottom of the trough was again 200 lux.

To produce shadows, black plates of various sizes were inserted into the light path at a position as near to the water surface as possible. Large ones were used for shading half of the trough, medium-sized ones for shading 2–4 arms and small ones for shading one arm.

RESULTS

I. *Direction of movements at light-dark boundary*

In order to reveal the basic pattern of behavior with respect to photic conditions, the starting direction of light- and dark-adapted animals was determined, using the directional index as set up in Figure 1. Experiments were performed using 21 specimens. After dark-adaptation for more than 4 hours, animals were exposed to light of 42 lux for fixed periods of various lengths. Directional indices were then determined by placing animals at the center of the trough, half of which was illuminated at a light intensity of 200 lux. Usually one or two animals did not move within the period of observation lasting 2 minutes. These were considered to be irresponsive and were discarded. Average directional indices obtained with the remaining individuals (usually 19 or more) are shown in Figure 2, curve A. Curve B shows percentages of animals which began to move only after 1 minute of illumination by the testing light. Three semi-circles inserted above are the actual directional patterns of fully dark-adapted animals and those after 2- and 16-minute light-adaptation, showing percentages of animals which moved towards the direction as indicated by the bars.

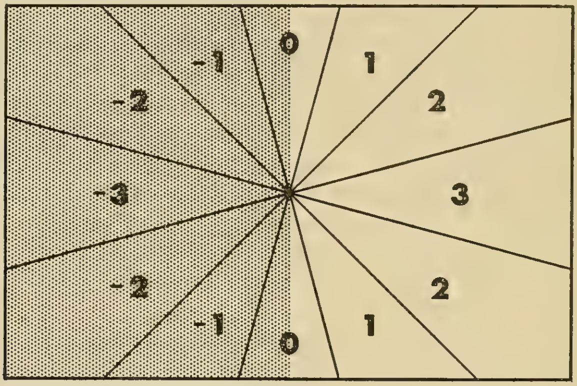

Figure 1. The method of determining the direction of movements (Method 1). The starfish was placed at the center. Shaded area is shown by dots. The figure in each section represents the directional index.

Thoroughly dark-adapted animals were rather inactive (curve B) and when they moved, the direction was more or less at random, as is shown by the directional pattern above, and the average directional index was nearly zero. However, all the animals which moved towards the −3 direction started within 1 minute whereas five out of six which moved to the +1 direction began to move much later. It is possible that the inactive specimens became partially light-adapted during the period of illumination by the test light whereas fully dark-adapted animals were photonegative.

As the duration of illumination was prolonged, animals became more active (curve B) and mostly moved into the illuminated area, as is shown by the two upper semi-circles on the right. The average directional index became well above 2 after 4-minute illumination (curve A).

When light-adapted animals were placed in darkness, they gradually lost their phototactic activity, the average directional indices becoming 1.3 and 0.8 after 20 minutes and 40 minutes, respectively.

II. *Determination of the starting direction*

The effect of light or shadow on the starting direction was studied using intact starfish as well as those from which all the ocelli had been removed. After light-

or dark-adaptation, they were brought into the center of the testing trough which was evenly lit. Before they showed any sign of locomotion, one arm was stimulated either by switching-on of the vertically placed microscope lamp with simultaneous extinction of the general illumination, or by casting a shadow produced by a small black plate. Results are shown in Figure 3. Here again, light-adaptation made animals photopositive so that more than 80% of the light-adapted animals started with the illuminated arm forward (A). A similar tendency was observed with eyeless specimens (C), though the positivity was less evident. Dark-adapted animals moved more or less randomly, regardless of the presence (B) or absence of ocelli (D).

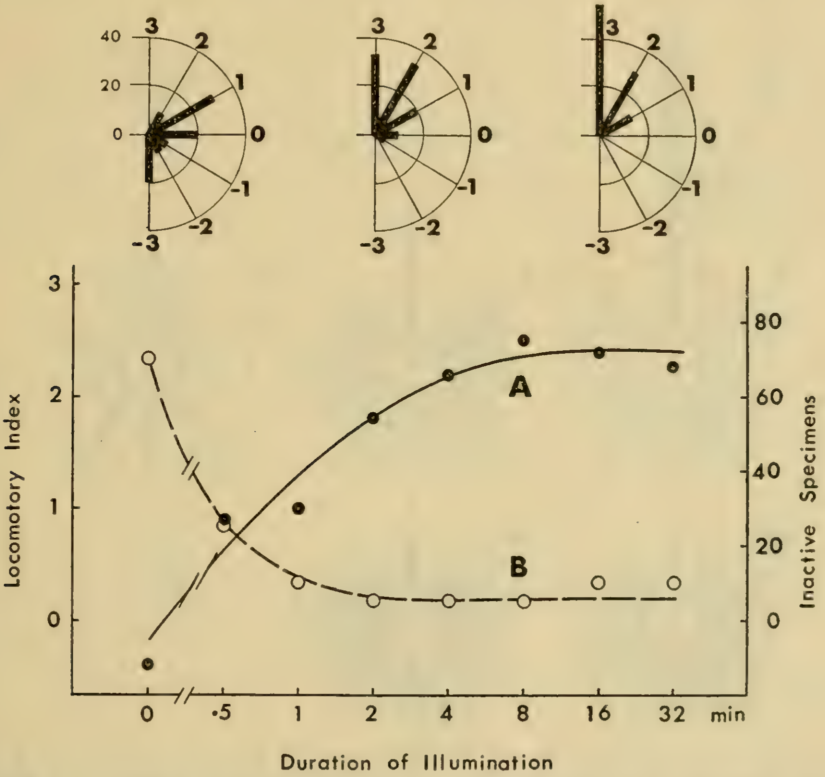

FIGURE 2. Changes in phototactic activity during light-adaptation. Abscissa; duration of illumination in minutes in logarithmic unit. Curve A: changes in average directional index, Curve B: percentages of animals which moved 1 minute after the beginning of stimulation. Semicircles inserted above show percentages of animals, as indicated by the lengths of bars, which moved in the corresponding direction.

If, on the contrary, one arm of light-adapted specimens was shaded (E), only a few (4%) moved towards the shaded side and a majority moved away from the shadow. It should be noted that a gradient existed in the locomotory pattern which increased with the distance from the shaded arm.

III. *Shifts of the leading arm*

The effect of shading on the direction of movements was examined using animals which were on the move. Moving starfish usually assume a characteristic posture. The leading arm (I) is stretched straight forward, towards which two side arms (II and V) bend, forming a fork shape. The two rear arms (III and IV) are slightly shortened with tips often lifting from the substratum. Hereafter, the arms II and V will be called lateral arms or the arms I, II and V, anterior arms, and the arms III and IV, posterior arms.

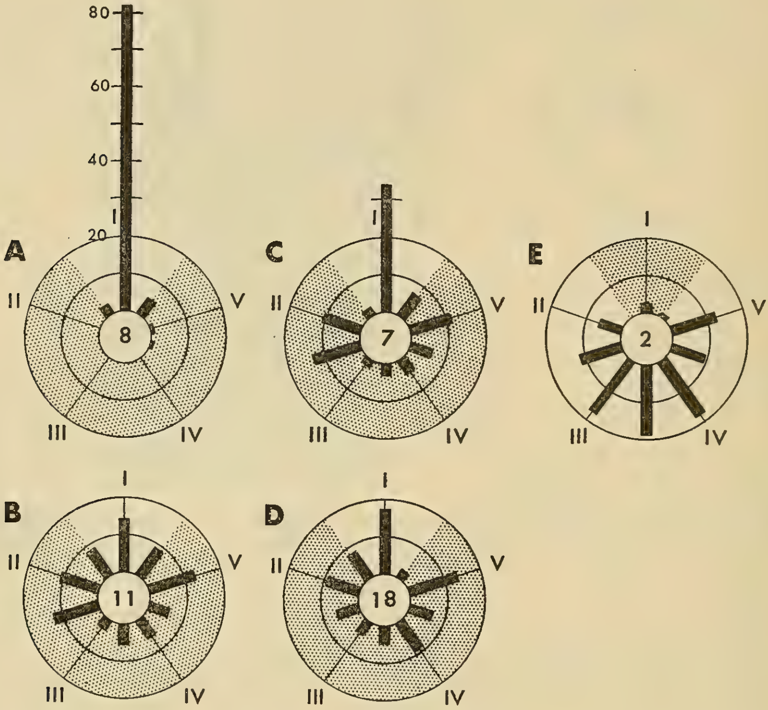

FIGURE 3. The direction of starting movements when an arm (I) was illuminated (A–D) or shaded (E). The lengths of bars represent percentages of animals which moved in the corresponding direction. Outer, middle and inner circles show respectively 20, 10 and 0%. A, C and E: light-adapted animals. B and D: dark-adapted animals. A, B and E: intact animals. C and D: eyeless animals. Number of experiments; A, 100; B, 135; C, 90; D, 88; E, 106.

Since it was found that the phototactic behavior was much disturbed by dark-adaptation, only light-adapted animals were used hereafter. It should be noted also that, although moving starfish usually kept the direction until they met some obstacles, responses to shadows were not simple but the direction sometimes altered in course of time. In the figures, therefore, the direction as observed at 15, 35 and 55 seconds after the beginning of stimulation is presented in order to produce a clearer image on the locomotory pattern.

(a) *Shading one or more arms of intact animals.* At first, a shadow was cast on one arm, namely, the leading or one of the lateral or the posterior arms, Results are shown in Figure 4.

When the leading arm was shaded (Fig. 4A), the locomotion usually stopped for a while. Another response to the shadow was seen at the tip of the stimulated arm which bent upward with a latency of 3–5 seconds. This reaction will be called "off" response. It took about 6–27 seconds for the animal to resume locomotion. During this period, the characteristic moving posture with the arm I forward also changed. Four unshaded arms (II–V) were all stretched and swung aimlessly while the off-response in the shaded arm was going on. Thus, if they moved at all within 15 seconds after shading, the first change in direction occurred at an almost equal frequency towards four arms which were left in light (upper row). Meanwhile, one of the lateral arms (II or V) became stretched straight forward, towards which the arms on both sides (I and III or I and IV) bent. The animals then began to move with the arm II or V forward (middle row). Once the new leadership was established, the locomotory direction was more or less fixed (lower row).

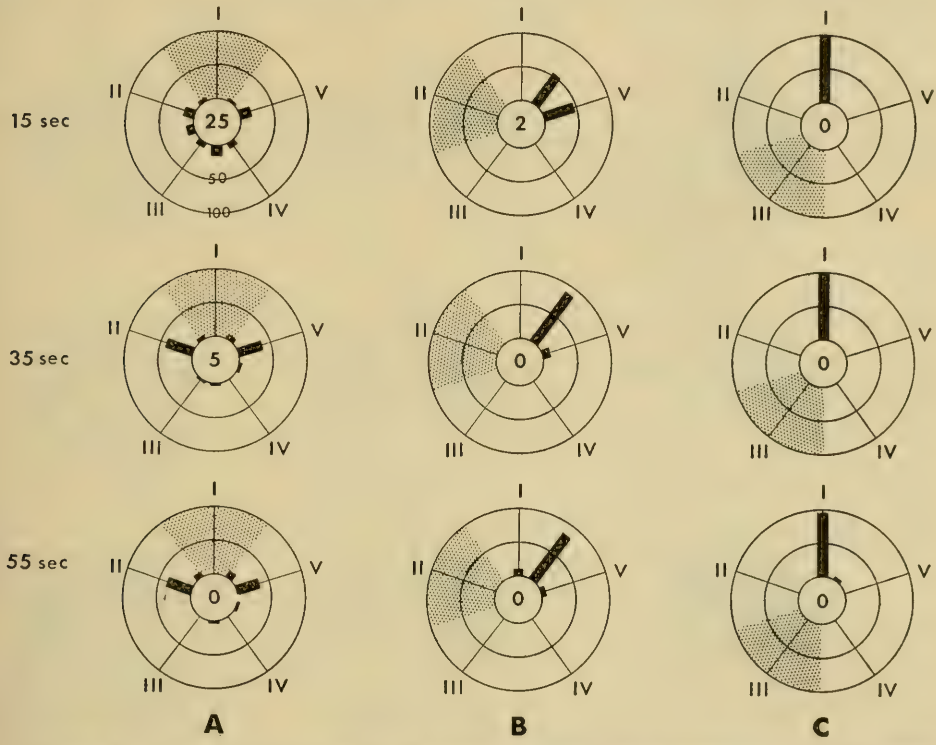

FIGURE 4. Changes in the direction of movement when one arm was shaded. The shaded arm is shown by dots. The lengths of bars represent percentages of animals which moved in the corresponding direction. Outer, middle and inner circles show respectively 100, 50 and 0%. Number of experiments: A, 44; B, 50; C, 30.

There were thus two phases of reactions, the initial and the later, in response to shadow. The initial reaction was a sort of "shock" reaction, lasting for a short period. The characteristic of the later reaction was a tendency of the leadership to shift back towards the original leading arm and it may be called "return" phase.

A similar tendency was seen when one of the lateral arms, for instance the arm II, was shaded (Fig. 4B). Here again, a small off-response appeared, though most of the animals did not stop. The opposite lateral arm, V, became soon stretched straight forward. At 5–13 seconds after shading, they moved towards either the arm V or the interradius between I and V (upper row). The return phase became apparent at 35 seconds, when 90% of the animals moved towards the direction of the interradius between I and V. The return process seemed to proceed still further in few animals so that the arm I sometimes resumed its leadership within 35 seconds (lower row).

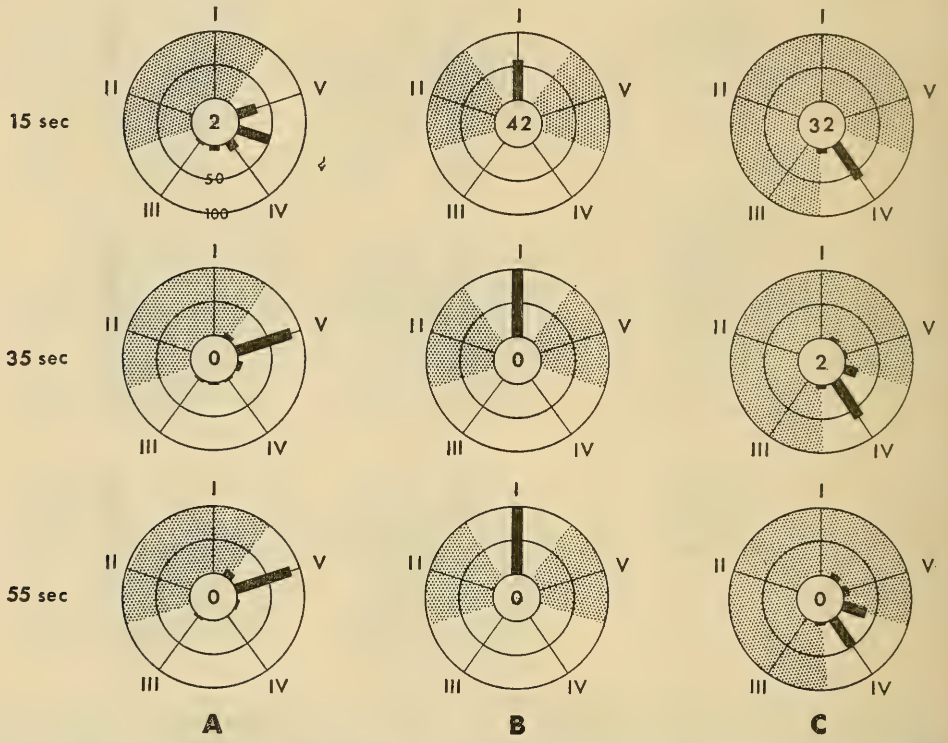

FIGURE 5. Changes in the direction of movement when more than one arm was shaded. The shaded arms are shown by dots. The way of presentation is the same as in Figure 4. Number of experiments: A, 50; B, 26; C, 50.

Casting a shadow onto one of the posterior arms, III or IV, caused no change in locomotory direction (Fig. 4C). It is important to note that in this case the off-response was not observed.

Clearer results were obtained when more than one arm was shaded simultaneously. Shading the leading, I, and one of the lateral arms, II, (Fig. 5A) changed the direction of locomotion in a manner as expected from the above-mentioned results. At 15 seconds after shading, about half of the specimens moved towards the interradius between the rays IV and V (upper row). They gradually changed the direction, moving finally with the arm V forward (middle and lower rows). The off-response was induced in both I and II arms.

When both lateral arms, II and V, were shaded simultaneously (Fig. 5B), the off-response was seen in both. More than 40% of the animals stopped for a moment (upper row) but soon resumed the original movements (middle and lower rows). Shading the arms IV and V produced essentially the same results as those obtained by shading the lateral arm V only. In other words, addition of a shadow on one of the posterior arms, in this case the arm IV, was without effect. It is interesting to note that the off-response was seen only in the arm V and not in the arm IV. As might be expected from the results mentioned above, it was in fact confirmed that upon simultaneous shading of the two posterior arms, animals neither changed their locomotion nor showed the off-response.

The results shown in Figures 4 and 5 indicate (*a*) that the effect of shading on changing the direction of movement is different in different arms, (*b*) that the initial reaction of changing direction usually occurs opposite to the shadow, and (*c*) that the direction of locomotion tends to return towards the original leading arm after the initial effect is over.

The return phase of the leadership was also seen when four arms were shaded (Fig. 5C). The off-response was observed only in the anterior three arms, I, II and V. Upon shading, animals stopped and the arm IV became stretched, towards which two arms on both sides, III and V, bent. If moved, the illuminated arm, IV, usually took the lead (upper row). At 35 seconds, though a majority still moved with the illuminated arm IV forward, a few others changed their direction towards the arm I (middle row). Such a tendency in changing direction was more evident at 55 seconds (lower row).

(b) *Photoreceptive site.* The possibility that the ocellus may function as a photoreceptive site was examined by comparing the behavior of intact animals with that of eyeless ones, both of which were on the move. Here, three arms were shaded simultaneously and results are shown in Figure 6. When anterior arms I, II and V were shaded (A), intact animals stopped for a while, the off-response appearing in all the three. The initial movement, if it occurred, took place mostly towards the interradius between the arms III and IV (upper row). Such posterior movements became gradually less evident (middle row) and at 55 seconds, locomotion took place even towards the shaded side in a few cases, though a majority moved with any one of the illuminated arms in advance (lower row).

In eyeless animals (Fig. 6B), although the same treatment produced hardly any change in the moving direction, the duration of stopping after shading was longer so that the percentage of animals which did not move at 15 seconds after shading was 24% (upper row). It may be added that the shaded arms were still capable of showing the off-response.

When arms I, IV and V were shaded (Fig. 6C), intact specimens usually turned to the interradius between II and III (upper row). The return phase was also evident so that the arm II finally took the lead in locomotion at high percentages (middle and lower rows).

Shadows cast on eyeless animals in the same way affected the locomotory direction only in few cases, where they moved mostly sideways (Fig. 6D upper row). The return action may have come into play also in this case, for percentages of the arm I taking the lead were much higher at 35 and 55 seconds (middle and lower rows) than at 15 seconds after shading.

These results strongly suggest that the ocelli do function as photoreceptive sites.

(c) *Effects of transection of the circumoral nerve ring.* The oral nerve ring may be considered to be a possible route through which the information concerning environmental changes could pass in order to induce coordinated movements in tube feet. This was studied by transecting the oral nerve ring at an interradius. Experiments were made by casting a shadow (1) on arm I (the same as Fig. 4A) with a cut between I and II, II and III or III and IV, (2) on arm II (the same as Fig. 4B) with a cut between II and III, III and IV or IV and V, (3) on arms I and II (the same as Fig. 5A) with a cut between I and II, II and III, III and IV, IV and V or V and I, (4) on arms I, IV and V (the same as Fig. 6C) with a cut between II and III, and (5) on arms I, II and V (the same as Fig. 6A) with a cut between I and II, III and IV or IV and V. The number of experiments in each case was 23–42. To avoid undue complication, only the results obtained with the last combination are presented in Figure 7.

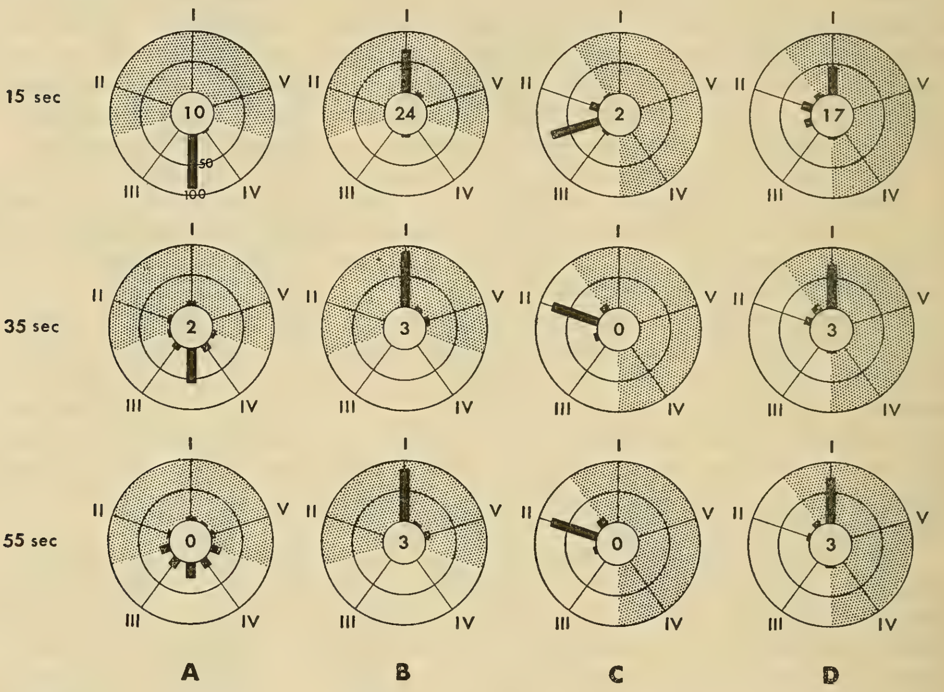

FIGURE 6. Comparison of the effect of shading three arms of moving specimens between intact and eyeless animals. The way of presentation is the same as in Figure 4. A and C: intact animals. B and D: eyeless animals. Number of experiments: A, 49; B, 29; C, 45; D, 29.

In order to perform experiments with such a variety in combinations as mentioned above, it was too inefficient to await fortuitous coincidence of the proper combination of the place of cut and the moving direction in accordance with the experimental planning. Since the place of cut was known, therefore, the arm which it was desired should take the lead was forced to do so by bending artificially the remaining four arms to assume the appropriate posture as well as by illuminating the leading arm so chosen. Once the leadership had been established as desired, animals were left under an evenly lit field for at least 1 minute before experiments.

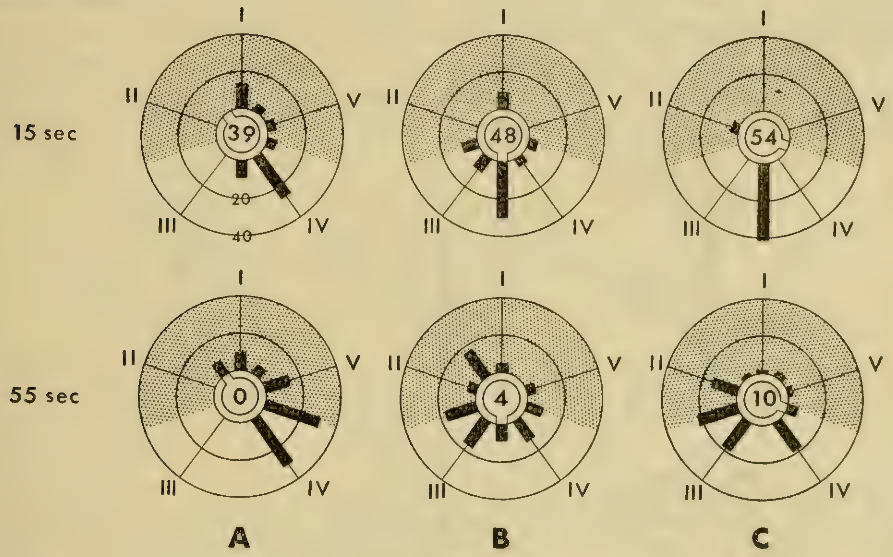

FIGURE 7. The effect of cutting the oral nerve ring on the direction of locomotion upon shading the anterior three arms. Outer, middle and inner circles represent respectively 40, 20 and 0%. The place of cut is indicated by the break on the inner double circle. Number of experiments: A and B, 23; C, 41.

Comparing Figure 7 with Figure 6A, which serves as a control, differences are found in two points. First, the number of animals which stopped upon shading was significantly larger in the operated animals. This might be due to disturbance in the pathway through which the initial shock reaction is conducted.

The second notable difference concerns the return phase. Thus the symmetrical pattern of the return process seen in intact animals (Fig. 6A lower row) was greatly disturbed if a cut was made between the arms I and II (Fig. 7A) or IV and V (Fig. 7C). The return process seems to have proceeded only anticlockwise from IV to I in the former case and clockwise from III to I in the latter case.

IV. *Effects of total shading on variously operated animals*

As has been shown in Figure 6, a partial shading exerted practically no effect on the direction of locomotion of moving animals if all the ocelli were removed. To study the relation between the ocellar function and the effect of shadow in more detail, a compound ocellus was removed from one arm, leaving the remaining four arms intact. The operated animals were brought into an evenly lit field. When the whole surface was shaded before they showed any sign of locomotion, they began to move with the eyeless arm forward without exception, resembling the results obtained by shading four arms of not-moving animals (Fig. 3A).

The work was extended using animals with ocelli removed from two, three or four arms. Results are shown in Figure 8. Three-eyed preparations moved towards the interradius between the two eyeless arms III and IV in the case of A and with either one of the eyeless arms (III or V) forward in the case of B.

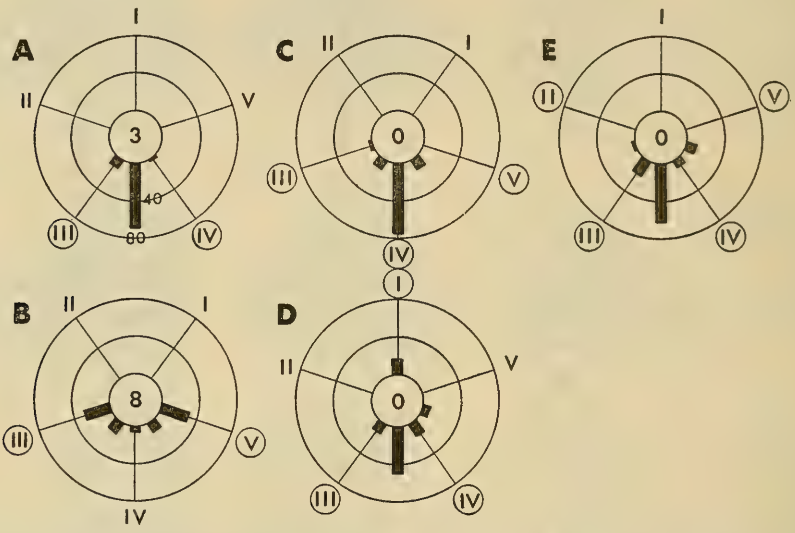

FIGURE 8. The starting direction upon shading the whole surface of three- (A and B), two- (C and D) and one- (E) eyed preparations. Outer, middle and inner circles represent respectively 80, 40 and 0%. Rays from which ocelli were removed are shown by the circle around the ray number. Number of experiments: A-D, 36; E, 35.

Similarly, two-eyed preparations moved towards the center of the three eyeless arms, namely, with the arm IV forward in the case of C and towards either the interradius of two adjacent eyeless arms III and IV or with the eyeless arm I forward in the case of D. One-eyed specimens moved mostly towards the center of the four operated arms, namely, the interradius between the arms III and IV (E). On the whole, operated animals tended to move with the operated arm(s) forward when the whole surface was shaded, the behavior resembling that of intact animals upon partial shading. These results strongly suggest that the shadow may be perceived only by intact arms.

Similar results were obtained by transecting the radial nerve in one ray near the proximal end. Upon shading, such preparations did not move towards the operated side, probably because the operated arm which could not coordinate with the rest of the arms may become an obstacle. However, the intact arms took the characteristic posture which looked as though the animals would start with the operated arm forward.

The above experiments appear to indicate that the starting direction is determined only by the arm(s) which are capable of perceiving the shadow through ocelli. If so, however, the locomotory pattern of one-eyed preparations (Fig. 8E) should have been the same as that obtained by shading one arm (Fig. 3E). This was not the case, suggesting that in intact animals, light received through ocelli is also effective in making the illuminated arm take the lead. In partially eyeless animals, such a subsidiary effect is concealed so that only the effect of shading becomes manifest.

A question then arises as to how the information obtained by intact arms is conducted to eyeless arms so as to cause their podia to step in a direction opposite to those of an intact arm when shaded. The whole surface of one-eyed preparations with the oral nerve ring transected was shaded (Fig. 9). The disturbance in locomotory pattern was more evident when cuts were made between the arms I and II (A) or II and III (B) than III and IV (C). This suggests that the two pairs of arms (II-V and III-IV) at symmetrical positions with respect to the intact arm I, received information at equal strength when the ring nerve was intact. Any disturbance in the conduction pathways will then result in an asymmetrical locomotory pattern.

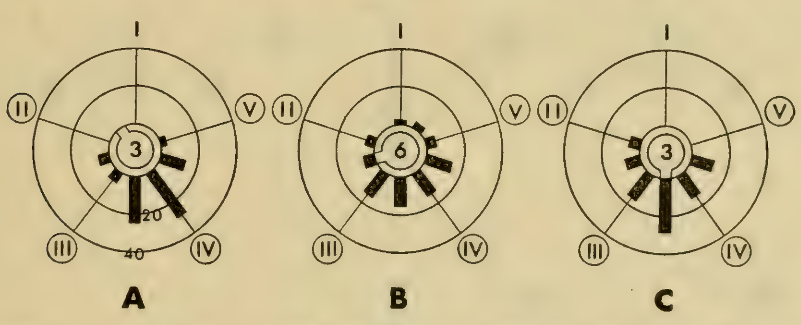

Figure 9. The effect of cutting the oral nerve ring on the direction of movement of one-eyed preparations upon total shading. Outer, middle and inner circles represent respectively 40, 20 and 0%. The place of cut is indicated by the break on the inner double circles. Number of experiments: 36 in each case.

The part played by the skin light sense was examined by removing an arm or arms near the central disk. When shaded, four-armed preparations always started with the operated region forward. Two arms on both sides of the operated ray took the typical advancing posture. Results obtained by shading the whole surface of three-, two-, and one-armed preparations (Fig. 10) showed the same tendency, namely, to move towards the radii from which arms had been removed. It must be pointed out that the locomotory patterns obtained by arm-removal (Fig. 10) are almost identical with those obtained by ocelli-removal (Fig. 8). It may be inferred from this that the dermal light sense which remains in eyeless arms plays only little, if any, role. The light appears therefore to have been received through ocelli.

One-armed preparations isolated from the central disk always moved with the base forward, the behavior resembling the response to shadow of one-armed preparations with the ring nerve attached (Fig. 10E). It seems possible that there may be a central mechanism at or near the junction of the radial nerve with the oral nerve ring. The mechanism may be identical with the so-called locomotory center (Mangold, 1908), and the activity of the center, whether it is excitatory or inhibitory, may be suppressed by the shadow as perceived through the compound ocellus, resulting in movements which, in direction, are the same as those of isolated arms.

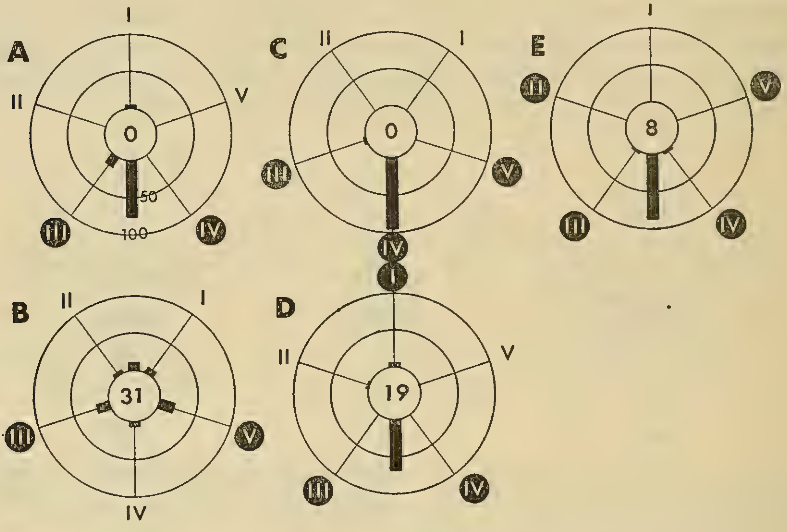

FIGURE 10. The starting direction upon shading the whole surface of three- (A and B), two- (C and D) and one- (E) armed preparations. Outer, middle and inner circles represent respectively 100, 50 and 0%. Removed rays are shown by black circles. Number of experiments: 36 in each case.

DISCUSSION

Locomotory behavior of starfishes has been studied widely (see Hyman, 1955). From such knowledge, major points which should be borne in mind for the type of experiments described above are the presence or absence of ray preference and persistent daily rhythmic activity. The starfish *Asterias amurensis* may show ray preference, for once they started to move, the direction was usually maintained for some time. However, such a dominance in one arm was not strong enough to resist environmental factors, such as an exposure to air for a short period or a decrease in light intensity. The type of daily rhythmic activity as reported by Rockstein and Spritzer (1960) was not observed in this species. Instead, they showed positive phototaxis at any time of the day so long as they were light-adapted. Negative geotaxis which is widely observed in starfishes was eliminated by careful arrangements of the experimental set up, using a water level.

Phototactic behavior in echinoderms depends generally on adaptive states. In the sea urchin, *Diadema antillarum*, a reversal of the sign of taxis occurs by altering adaptive states; dark-adapted animals are photonegative and light-adapted ones are photopositive (Millott, 1954). Mangold (1909) and van Weel (1935) have described how asteroids reverse the direction of their responses to light, which depend on the light intensity; they are photopositive under dim light but negative under bright light. The light-induced locomotion of the present species was affected by the state of adaptation as observed in the sea urchin, *Temnopleurus toreumaticus* (Yoshida, 1966). Thus, in both species, the phototactic behavior is evident only when they have been light-adapted. It is not certain, however, whether the animals become indifferent to light during the course of dark-adaptation or whether the process of light-adaptation proceeds too fast for the present method to reveal a dark-adapted state, during which the animals were photonegative. As is shown in Figure 2, the process was indeed rather fast, being complete in a few minutes.

Apart from the work of Hartline, Wagner and MacNichol (1952), evidence as to the photoreceptive function of ocelli of starfishes is inconclusive (Yoshida, 1966). The present work has revealed that it does function in *Asterias amurensis*. Thus, percentages of animals which started with an illuminated arm in advance were significantly higher in intact animals (Fig. 3A) than in eyeless ones (Fig. 3C) and the direction of movement could not be changed by casting a shadow onto three arms of eyeless animals (Fig. 6B and D). Also animals lacking one or more eyes moved with the operated rays in advance when the whole surface was shaded (Fig. 8), the behavior simulating that when only the intact arms were shaded. One might infer from this that the shadow and not the light as perceived through ocelli is the determining factor in changing locomotory direction. This seems to be not entirely true. If so, the starting direction of intact animals when one arm is shaded (Fig. 3E) should have been the same as that of the one-eyed specimens (Fig. 8E). The fact was that in the former case, movements took place towards any of the illuminated rays, whereas in the latter case, a majority moved towards the interradius between the two arms farthest from the intact one. It appears, therefore, that light as well as shade is perceived through ocelli, the light causing animals to move towards it and the shadow, away from it. Indeed, when illuminated, one- and three-eyed preparations moved with the intact rays forward.

Though the photoreceptive function of ocelli is thus clear, the fact that eyeless animals were slightly more photopositive when light-adapted (Fig. 3C) than when dark-adapted (Fig. 3D) would suggest that the ocelli are not the sole photoreceptors involved but that an extra-ocellar system may also function. However, as the behavior of specimens lacking arms (Fig. 10) resembled that of those without ocelli (Fig. 8), the extra-ocellar sense may play only a minor role, appearing to be significant only when all the ocelli were removed. Indeed, two kinds of photoreceptive systems, ocellar and an extra-ocellar system which appears diffuse, have been distinguished in the same species by the study of the action spectrum for inducing reflex reactions in arm tips, and the sensitivity of the latter was found to be considerably lower than that of the former (Yoshida and Ohtsuki, 1966).

The relation of the mechanism of inducing the off-response to that involved in controlling the locomotory direction is not certain. When animals were not moving, the off-response was seen in all the five arms and the animals were induced to start moving with any one ray forward when properly stimulated (Fig. 3A). Again, moving animals changed direction when a shadow was cast on arm(s) which were capable of showing the off-response, namely, the anterior three arms (Fig. 4A, B, Fig. 5A and Fig. 6A), and extending a shadow onto one of the posterior arms produced practically no effect (see Fig. 4C and compare Fig. 5A with Fig. 6C). However, if shading one of the posterior arms is without effect, the locomotory pattern obtained by shading the anterior three arms (Fig. 6A) should have been the same as that obtained by extending the shadow onto one of the posterior arms (Fig. 5C). This was not the case. It may be suggested therefore that light falling on the posterior arms is also effective in inducing animals to move towards it, if the dominance in the leadership of the anterior arms is abolished by shadows.

It has now become possible, on the one hand, not only to induce the starfish to start with any one ray forward as desired (Fig. 3A) but also to shift the leading arm to either one of the lateral arms (Fig. 5A and Fig. 6C), to either one of the posterior arms (Fig. 5C), or to let them move backward (Fig. 6A) by a simple combination of light and shadow stimuli, though in the latter two cases the new direction does not last long. Again, the role of ocellus as a photoreceptor, the radial nerve as a conduction pathway and the circumoral nerve ring as a central mechanism have been confirmed. It must be admitted, however, that the intimate mechanism involved in controlling the locomotory direction is rather complex and labile. Reviewing the work on the directional coordination of tube feet stepping, Smith (1965) held the view of "one-arm dominance" rather than a "five-ray interaction." The exact meaning of "dominance" has not yet been defined physiologically but in order to explain the present results, a reconciliation of the two theories seems to be required.

However complex and labile though it may be, the effect of shading on changing the moving direction was different in different arms in a predictable manner (Figs. 4, 5 and 6A and C) and there was a gradient in the locomotory pattern of starting movements when one arm was shaded (Fig. 3E). That the direction is determined not in a haphazard manner would mean that there exists a rule, namely, a "gradient" among the five centers in susceptibility to environmental changes as well as in the relative strength of their effects on the activity of other centers. The asymmetrical locomotory pattern obtained by transecting the oral nerve ring (Figs. 7 and 9) would support the possibility of mutual interaction among the centers.

Such a concept of "gradient" may help to interpret the return phase (Figs. 4B, 5A and C, and 6A and C). The effect of shadow may be short-lasting and not strong enough to abolish the existing gradient completely so that when the initial reaction of changing direction, which usually occurs opposite to the shadow (Figs. 5C and 6A and C), is over, a more or less pre-established gradient may become effective again and the direction of movement tends to return towards the original leading arm. The differences seen between stationary and moving animals under identical conditions of stimulation (compare Fig. 3A with Fig. 5C and Fig. 3E with Fig. 4A) may be accounted for by the same reasoning.

Finally, the posture of the arms seems to be important for determining locomotion, for animals change the posture mostly before changes in locomotory direction.

Also, if it is desired to induce movements with a certain ray leading, shaping the arms so that they assume the typical posture, leads to a high degree of success. But in some cases, such changes in posture were also observed after changes in direction. Therefore, it is difficult to decide whether changing the posture, hence the asymmetrical tension imposed on sides of the lateral arms, is the cause of the change in direction or whether the posture may be assumed by a common mechanism which also governs the "gradient in dominancy."

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SUMMARY

1. The starfish, *Asterias amurensis,* was positively phototactic when light-adapted.

2. Shading four arms caused animals to start moving with the illuminated ray forward. The direction of changes in locomotion of moving animals was highly predictable when the animals were properly stimulated by partial shading.

3. The response of changing direction involved two phases, the initial and the later. In the initial phase, animals tended to move opposite to the shaded arm(s) and in the later phase, the direction gradually returned towards the original leading arm.

4. When moving, animals assumed a typical advancing posture. Shadows cast on them induced off-responses in the anterior three arms, but when animals were stationary, the off-response appeared in all the five arm tips. The relation of these reactions with the changing direction has been discussed.

5. Experiments using preparations lacking ocelli or arms, as well as those with the radial nerve transected, suggested that the apparent positive phototaxis was induced mainly by shadows which were perceived through ocelli. Light seemed to play a secondary role. The function of an extra-ocellar light sense was recognized, but it appeared only when all the ocelli were removed.

6. The central mechanism involved in controlling the locomotory direction has been discussed.

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INDEX

A

Abalone, banding patterns in, 139.
Abarenicola, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Abnormalities of development in cynthia silkworms after ecdysone treatment, 344.
Acanthodoris, morphology of digestive system of, 305.
Acartia, effect of xanthene dyes on phototaxis of, 398.
Acheta, pheromone-like dispersant affecting distribution of, 490.
  use of for assay of insect hormone-like materials, 154.
Acid glycoprotein of sea urchin egg, 425.
  mucopolysaccharides in refractile bodies of Childia sperm, 382.
Acoel turbellarian sperm, refractile bodies in, 382.
Actinomycin D, effects of on furrow induction in Arbacia eggs, 356.
Action of ecdysone on cynthia silkworms, 344.
  spectra, photosynthetic, of endozoic algae, 411.
Activity cycles in Ameiva, 325.
Adaptive significance of air-gaping by Modiolus, 60.
  of Callianassa oxygen perception, 261.
Adult chicken spleen as source of growth-stimulating factor for embryonic spleen grafts, 473.
d'Agostino, A. S., and L. Provasoli. Effects of salinity and nutrients on mono- and diaxenic cultures of two strains of Artemia, 1.
Aggregation of slime molds, chemotaxis as factor in, 298.
Ahearn, G. A. A comparative study of P-32 uptake by whole animals and isolated body regions of the sea cucumber Holothuria, 367.
Air-gaping by Modiolus, 60.
Albedo response of chromatophores in Uca, 221.
Algae, endozoic, photosynthetic capacities of, 411.
Algal cells, feeding on by Nassarius veligers, 481.
Ameiva, gonadal-fat body relationship in, 325.
Amino acid levels in polychaete coelomic fluid and body wall, 35.
Amino acids, incorporation of by unfertilized sea urchin eggs, 209.
  in Arbacia egg fertilization product, 425.
  of Polychaetes, effect of osmotic dilution on, 252.
Amphibian, sialic acid in egg capsules and oviducts of, 266.
Amphipod commensal of starfish, 148.
Amphitrite, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Analogues of ecdysone, assay of on cynthia silkworm, 344.
Analysis of Arbacia egg surface glycoproteins, 425.
Anatomy of Magelona, 272.
  of Microphallus larvae, 332.
  of nudibranch digestive system, 305.
Annelid, morphology, feeding, and behavior of, 272.
  pelagic larvae of, 503.
  responses of to osmotic stress, 118.
Annelids, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on amino acids of, 252.
Aphrodite, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Appendages, role of in feeding and swimming of Conchoecia, 456.
Arbacia egg glycoproteins, 425.
  eggs, furrow induction in, 356.
Arme, C. Effect of the plerocercoid larva of a pseudophyllidean cestode, Ligula, on the pituitary gland and gonads of its host, 15.
Artemia, effects of salinity and nutrients on cultures of, 1.
Ascidia, cardiovascular physiology of, 434.
Asexual generations of Microphallus, 332.

Assay of ecdysone and analogues on cynthia silkworms, 344.
Asterias, characteristics of commensal of, 148.
  phototactic behavior of, 516.
Attractant substance affecting distribution of Acheta, 490.
Atyid shrimp, osmoregulation in, 235.
Atz, J. W. See B. Lofts, 74.

B

Background, responses of Uca chromatophores to, 221.
Bacteria, role of in P-32 uptake of holothurian, 367.
Banding patterns in Haliotis, 139.
Barnwell, F. H. Comparative aspects of the chromatophoric responses to light and temperature in fiddler crabs of the genus Uca, 221.
Behavior, phototactic, of Asterias, 516.
  thermoregulatory, in Ameiva, 325.
  of Caprella, 148.
  of Magelona, 272.
Behavioral studies on Acheta, 490.
Benzoic acid derivatives, juvenile hormone activity of, 154.
Björnberg, T. K. S., and K. M. Wilbur. Copepod phototaxis and vertical migration influenced by xanthene dyes, 398.
Blood of Magelona, 272.
  osmotic pressure of caridean shrimp, 235.
  picture in x-irradiated mice, 320.
Body wall of polychaetes, amino acids in, 35.
Born, J. W. Osmoregulatory capacities of two caridean shrimps, Syncaris and Palaemon, 235.
Breeding season of Spio, 503.
Brine shrimp, effects of salinity and nutrients on, 1.
Bryozoan as food for nudibranch, 305.
Burrowing activity in Magelona, 272.
  in Penaeus, 48.
  shrimp, external oxygen perception by, 261.

C

Calanopia, effect of xanthene dyes on phototaxis of, 398.
Callianassa, external oxygen perception in, 261.
Campbell, R. D. Holdfast movement in the hydroid Corymorpha, 26.
Campbell, R. D., and F. Campbell. Tubularia regeneration: Radial organization of tentacles, gonophores and endoderm, 245.
Caprella, feeding, behavior and host specificity of, 148.
Cardiovascular physiology of tunicates, 434.
Caridean shrimps, osmoregulation in, 235.
Case, J. F. See R. D. Farley, 261.
Cell density, effect of on aggregation of slime molds, 298.
  distribution in elongation of hydroid holdfast, 26.
  division of diatom, effect of phosphate concentration on, 199.
  layers in hydra, reversal of, 465.
Cellular slime molds, chemotaxis in, 298.
Centrifugation of Arbacia eggs under pressure, 356.
Cercaria of Microphallus, 332.
Cestode larva, effects on on host pituitary and gonads, 15.
Chaetoceros, effect of phosphate concentration on yield of, 199.
Chelyosoma, cardiovascular physiology of, 434.
Chemical analysis of Arbacia egg glycoproteins, 425.
  constitution of insect hormone-like materials, 154.
Chemotaxis in cellular slime molds, 298.
Chick spleen grafts, growth stimulation of, 473.
Childia, refractile bodies in sperm of, 382.
Chloride concentrations in caridean shrimp, 235.
  movements in Nereis, 118.
Chlorophyll in endozoic algae of Favia, 411.
Chorio-allantoic grafts of spleen in chick embryo, 473.
Chromatophoric responses of Uca, comparative study of, 221.
Ciona, cardiovascular physiology of, 434.
Circulatory system of Magelona, 272.
Cladoceran, photoperiod control of winter diapause in, 176.
Clark, M. E. Free amino-acid levels in the coelomic fluid and body wall of polychaetes, 35.
  a survey of the effect of osmotic dilution on free amino acids of various polychaetes, 252.
Cleavage furrow induction in Arbacia eggs, 356.
Coelenterate, photosynthetic capacities of endozoic algae of, 411.
  regeneration in, 245.
  reversal of cell layers in, 564.
  stolon, growth in, 26.
Coelomic fluid of polychaetes, amino acid levels in, 35.
Cohen, J. D. See J. A. Stevenson, 160.
Colcemid, effects of on furrow induction in Arbacia eggs, 356.
Cold, effect of on chromatophore responses of Uca, 221.
  on testicular regression in hypophysectomized Fundulus, 74.

Color changes in fiddler crabs, comparative study of, 221.
Commensal of starfish, feeding, behavior, and specificity of, 148.
Comparative P-32 uptake by Holothuria, 367.
  study of chromatophore response of Uca, 221.
Concentration of phosphate, effects of on cell division of diatom, 199.
Conchoecia, feeding and swimming of, 456.
Conditioning of areas by crickets, 490.
Control of winter diapause in Daphnia, 176.
Copepod phototaxis, as influenced by xanthene dyes, 398.
Coral, photosynthetic capacities of endozoic algae of, 411.
Corella, cardiovascular physiology of, 434.
Cornin, effects of on furrow induction in Arbacia eggs, 356.
Corymorpha, holdfast movement in, 26.
Cortisol, effect of on testicular regression in hypophysectomized Fundulus, 74.
Crab, horseshoe, parasite of, 332.
Crabs, fiddler, comparative study of chromatophores of, 221.
Crayfish, proecdysial growth and developmental processes in, 160.
Cricket, pheromone-like dispersant affecting distribution of, 490.
Criteria for molt stages in crayfish, 160.
Crustacean, effects of salinity and nutrients on mono- and diaxenic cultures of, 1.
  external oxygen perception by, 261.
  feeding and swimming of, 456.
  osmoregulation in, 235.
  photoperiod control of winter diapause in, 176.
  proecdysial growth and developmental processes in, 160.
Cuticle formation in crayfish, 160.
  in cynthia silkworms after ecdysone treatment, 344.
Cycle, diurnal, in copepod migration, 398.
Cycles, gonadal, in lizards, relation of to fat body, 325.
Cyclotella as food for Nassarius veligers, 481.
Cynthia silkworm pupae, action of ecdysone and analogues on, 344.
Cyprinodont fish, effects of cold and cortisol on testicular regression in, after hypophysectomy, 74.
Cytochemistry of refractile bodies in Childia sperm, 382.
Cytokinesis inhibitors, effects of on furrow induction in Arbacia eggs, 356.
Cytology of testes of hypophysectomized Fundulus, 74.

D

Daphnia, photoperiod control of winter diapause in, 176.
Darkness, in relation to migration of copepods, 398.
  role of in control of winter diapause in Daphnia, 176.
    in emergence of Penaeus from substrate, 48.
Density of cells, effect of on chemotaxis in slime molds, 298.
Desiccation, responses of Siphonaria to, 96.
Deuterium oxide, effects of on furrow induction in Arbacia eggs, 356.
Developing chick spleen grafts, growth stimulation in, 473.
  mice, recovery of from x-irradiation, 320.
  spermatozoa of Childia, refractile bodies in, 382.
Development of regenerating tissues in Tubularia, 245.
Developmental effects of ecdysone and analogues on cynthia silkworms, 344.
  processes in crayfish, 160.
Diapause in Daphnia, photoperiod control of, 176.
Diapausing pupae of cynthia silkworm, action of ecdysone and analogues on, 344.
Diatom, effect of phosphate concentration on yield of, 199.
Diaxenic cultures of Artemia, effects of salinity and nutrients on, 1.
Dictyostelium, chemotaxis in, 298.
Diemyctilus, sialic acid in egg capsules and oviducts of, 266.
Digenetic trematode, life-cycle and systematics of, 332.
Digestive system of Acanthodoris, morphology of, 305.
Dilution, osmotic, effect of on free amino acids of polychaetes, 252.
Diopatra, amino acids in coelomic fluid and body wall of, 35.
Directional movements of starfish, 516.
Dispersant affecting distribution of Acheta, 490.
Distribution of Acheta, pheromone-like dispersant affecting, 490.
Diurnal migratory cycle in copepods, 398.
  pattern of burrowing behavior in Penaeus, 48.
Dodson, A. N. See W. H. Thomas, 199.
Dunaliella as food for Nassarius veligers, 481.
Dyes, xanthene, influence of on copepod phototaxis, 398.

E

Ecdysone and analogues, assay of on cynthia silkworms, 344.
Echinoderm, P-32 uptake by, 367.
  phototactic behavior of, 516.
  commensal, characteristics of, 148.
  egg glycoproteins, 425.
  eggs, furrow induction in, 356.
    unfertilized, protein synthesis in, 209.
Ecological significance of chromatophore response in Uca, 221.
Ecology of caridean shrimp, 235.
  of Modiolus, 60.
  of Nereis, 118.
Effects of cestode larvae on pituitary gland and gonads of host, 15.
  of chemical agents on furrow induction in Arbacia eggs, 356.
  of low temperature on testicular regression in hypophysectomized Fundulus, 74.
  of phosphate concentration on cell division in diatom, 199.
  of salinity on Artemia, 1.
Egg capsules of newt, sialic acid in, 266.
  surface glycoproteins of Arbacia, 425.
Eggs, unfertilized, of sea urchin, protein synthesis in, 209.
  of Arbacia, furrow induction in, 356.
Electrical properties of tunicate heart, 434.
Electron microscopy of refractile bodies in Childia sperm, 382.
Elongation, mechanism of in hydroid, 26.
Embryonic spleen graft in chick, growth stimulation of, 473.
Embryos, mouse, hematological recovery of, following x-irradiation, 320.
Emergence of Penaeus from substrate, factors affecting, 48.
Endocrine activity of ecydysone and analogues, 344.
Endocrinological relations in lizard, 325.
Endoderm, reorganization of in Tubularia regeneration, 245.
Endozoic algae of Favia, photosynthetic capacities of, 411.
Epiphragm of snails, permeability of to water, 87.
Erythrophore responses of Uca, 221.
Estradiol, effect of on fat body of lizards, 325.
Estuarine polychaete, responses of to osmotic stress, 118.
Eunice, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.

F

Factors affecting emergence of Penaeus from substrate, 48.
False limpet, responses of to osmotic stress, 96.
Farley, R. D., and J. F. Case. Perception of external oxygen by the burrowing shrimp, Callianassa, 261.
Fat body-gonadal relationship in lizards, 325.
Favia, photosynthetic capacities of endozoic algae of, 411.
Fecundity of Artemia, effects of salinity on, 1.
Feeding on algal cells by Nassarius veligers, 481.
  habits of Caprella, 148.
  of Magelona, 272.
  of nudibranch, 305.
  rhythm in Penaeus, 48.
  and swimming of Conchoecia, 456.
Fertilization of newt eggs, possible role of sialic acid in, 266.
  product of Arbacia eggs, analysis of, 425.
Fetal x-irradiation, hematological recovery of mice after, 320.
Fiddler crabs, comparative study of chromatophores of, 221.
Fish, effect of cold and cortisol on testicular regression in, after hypophysectomy, 74.
  parasite, effects of on host pituitary and gonads, 15.
Fisher, F. M. See R. O. McAlister, 96.
Flatworm sperm, refractile bodies of, 382.
Fluorescein, effects of on copepod phototaxis, 398.
p-Fluorophenylalanine, effects of on furrow induction in Arbacia eggs, 356.
Free amino acids in polychaete coelomic fluid and body wall, 35.
  of polychaetes, effect of osmotic dilution on, 252.
Freeman, S. B. See A. A. Humphries, Jr., 266.
Functional morphology of nudibranch digestive system, 305.
Fundulus, effect of cold and cortisol on testicular regression in, after hypophysectomy, 74.
Furrow induction in Arbacia eggs, effects of chemicals on, 356.

G

Galleria, use of for assay of insect hormone-like materials, 154.
Gastrolith formation in crayfish, 160.
Gastropod, responses of to osmotic stress, 96.
  larvae, selective feeding of on algae, 481.
Glycoproteins in egg surface of Arbacia, 425.
Gonadal-fat body relationship in lizards, 325.

Gonads of host, effects of cestode larva on, 15.
Gonophores, regeneration of in Tubularia, 245.
Graft, spleen, in chick, growth stimulation of, 473.
Growth and developmental processes in crayfish, 160.
  rates in Haliotis, 139.
  stimulation in chick spleen grafts, 473.
GUCKERT, R. H. See J. R. STEVENSON, 160.

H

Haliotis, banding patterns in, 139.
HALLDAL, P. Photosynthetic capacities and photosynthetic action spectra of endozoic algae of the massive coral Favia, 411.
Heart physiology of tunicates, 434.
Heat, effect of on chromatophore responses of Uca, 221.
Helix, permeability of epiphragm of to water, 87.
Hematological recovery of mouse after fetal x-irradiation, 320.
HENLEY, C. Refractile bodies in the developing and mature spermatozoa of Childia and their possible significance, 382.
HESS, E. H. See O. J. SEXTON, 490.
HILL, J. C. See R. G. STROSS, 176.
Histology of Magelona, 272.
  of nudibranch digestive system, 305.
  of parasitized fish, 15.
  of regenerating hydra, 465.
Histochemistry of nudibranch digestive system, 305.
Holothuria, P-32 uptake by, 367.
Hormone activity, insect, of natural and synthetic materials, 154.
  effects on cynthia silkworm pupae, 344.
Horseshoe crab, parasite of, 332.
Host pituitary and gonads, effect of cestode larva on, 15.
  specificity of commensal, 148.
HUGHES, D. A. Factors controlling emergence of pink shrimp from the substrate, 48.
HUMPHRIES, A. A. JR., S. B. FREEMAN AND W. M. WORKMAN. Sialic acid in egg capsules and oviducts of the newt Notophthalmus, 266.
Hydra, reversal of cell layers in, 465.
Hydroid, holdfast movement in, 26.
  regeneration in, 245.
Hypophysectomized Fundulus, effect of cold and cortisol on testicular regression in, 74.

I

Inclusions in spermatozoa of Childia, 382.
Induction of furrows in Arbacia eggs, effects of chemicals on, 356.
Inhibitors, mitotic, effects of on furrow induction in Arbacia eggs, 356.
Insect hormone activity in natural and synthetic materials, 154.
Intensity of light, effect of on chromatophore responses of Uca, 221.
Ionizing radiation, recovery of mouse embryos from, 320.
ISHIHARA, K. Chemical analysis of glycoproteins in the egg surface of the sea urchin Arbacia, 425.
Isolated body regions of holothurian, P-32 uptake by, 367.

J

Jelly coat of Arbacia egg, analysis of, 425.
  layer of newt egg, sialic acid in, 266.
JONES, M. L. On the morphology, feeding, and behavior of Magelona, 272.
Juvenile hormone activity of benzoic acid derivatives, 154.

K

KONIJN, T. M. Chemotaxis in the cellular slime molds, II, 298.
KRIEBEL, M. E. Studies on cardiovascular physiology of tunicates, 434.

L

Larva, cestode, effect of on pituitary and gonads of host, 15.
Larvae, pelagic, of Spio, 503.
  of Nassarius, selective feeding of on algae, 481.
Layers of cells in hydra, reversal of, 465.
LENT, C. M. Air-gaping by the ribbed mussel Modiolus; effects and adaptive significance, 60.
Leptinotarsa, use of for assay of insect hormone-like material, 154.
Leucophore responses of Uca, 221.
Leukocytosis in x-irradiated mice, 320.
Life-cycle of Microphallus, 332.
Light, effect of on Uca chromatophores, 221.
  reaction of Asterias to, 516.
  responses of copepods, effect of xanthene dyes on, 398.
    of endozoic algae of Favia to, 411.
  role of in control of winter diapause in Daphnia, 176.
    in emergence of Penaeus from substrate, 48.
Ligula larva, effects of on pituitary and gonads of host, 15.
Limpet, responses of to osmotic stress, 96.
Limulus, life-cycle of parasite of, 332.
Lipid storage in lizards, relationship of to gonads, 325.

Lizards, gonadal-fat body relationship in, 325.
Lochhead, J. H. The feeding and swimming of Conchoecia, 456.
Locomotion of Conchoecia, 456.
Locomotor activity of copepods, effect of xanthene dyes on, 398.
  of starfish, as affected by light, 516.
Locusta, use of for assay of insect hormone-like materials, 154.
Lofts, B., G. E. Pickford and J. W. Atz. The effects of low temperature, and cortisol, on testicular regression in the hypophysectomized cyprinodont fish, Fundulus, 74.
Longo, J. See A. M. Mun, 473.
Loss of water from snails, 87.
Low temperature, effect of on testicular regression in hypophysectomized Fundulus, 74.
Lytechinus eggs, unfertilized, protein synthesis in, 209.

M

Machin, J. The permeability of the epiphragm of terrestrial snails to water vapor, 87.
Macklin, M. Reversal of cell layers in hydra: A critical re-appraisal, 465.
McAlister, R. O., and F. M. Fisher. Responses of the false limpet, Siphonaria, to osmotic stress, 96.
Mechanical properties of tunicate heart, 434.
Mechanism of holdfast elongation in hydroid, 26.
Melanophore responses of Uca, 221.
Membrane filter barrier, use of in demonstration of growth-stimulating factor in chick spleen grafts, 473.
Mercaptoethanol, effects of on furrow induction in Arbacia eggs, 356.
Metabolic inhibitors, effects of on furrow induction in Arbacia eggs, 356.
Metamorphosis of Spio larvae, 503.
Microphallus, life-cycle of, 332.
Migration of copepods, effect of xanthene dyes on, 398.
Mitosis of diatom, effects of phosphate concentration on rate of, 199.
Mitotic furrow induction in Arbacia eggs, 356.
Modiolus, air-gaping by, 60.
Mollusc, banding patterns in, 139.
  digestive system, morphology of, 305.
Molting stages of Orconectes, 160.
Monaxenic strains of Artemia, effects of salinity and nutrients on, 1.
Morphology of Magelona, 272.
  of Microphallus, 332.
  of nudibranch digestive system, 305.
  of refractile bodies in Childia sperm, 382.
Morse, M. P. Functional morphology in the digestive system of the nudibranch mollusc Acanthodoris, 305.
Mouse, hematological recovery of, following x-irradiation, 320.
Movement of holdfast in Corymorpha elongation, 26.
  of starfish in response to light, 516.
Mun, A. M., and J. Longo. Growth stimulation of embryonic spleen graft by adult chicken spleen across a membrane barrier, 473.
Murakami, T. H. See S. B. Zimmerman, 356.
Musculature of Magelona, 272.
Mussel, air-gaping by, 60.
Myxamoebae, chemotaxis in, 298.

N

Nainereis, amino acids in coelomic fluid and body wall of, 35.
Nassarius larvae, selective feeding of on algae, 481.
Natural and synthetic materials with insect hormone activity, 154.
Neoamphitrite, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Neodysdercus, use of for assay of insect hormone-like materials, 154.
Nephtys, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Nereis, amino acids of, 35, 252.
  responses of to osmotic stress, 118.
Nereocystis, annual cycle of, as related to banding pattern of Haliotis, 139.
Nervous system of Magelona, 272.
Newt, sialic acid in egg capsules and oviducts of, 266.
Notophthalmus, sialic acid in egg capsules and oviducts of, 266.
Nudibranch digestive system, morphology of, 305.
Number of cells, role of in chemotaxis of slime molds, 298.
Nutrients, effects of on Artemia, 1.
Nutrition of Nassarius veligers, 481.

O

Occurrence of pelagic larvae in Spio, 503.
Ocelli of starfish, role of in light perception, 516.

Ohtsuki, H. See M. Yoshida, 516.
Oglesby, L. C. Responses of an estuarine population of the polychaete Nereis to osmotic stress, 118.
Olsen, D. Banding patterns of Haliotis as indicators of botanical and animal succession, 139.
Oocytes of newt, assay of for sialic acid, 266.
Orchidectomy, effect of on fat body of lizard, 325.
Orconectes, proecdysial growth and developmental processes in, 160.
Organization of Tubularia tissues during regeneration, 245.
Orientation of cell layers in hydra, 465.
Osmoregulatory capacities of caridean shrimps, 235.
Osmotic dilution, effect of on free amino acids of polychaetes, 252.
  stress, responses of Nereis to, 118.
    of Siphonaria to, 96.
Ostracod, feeding and swimming in, 456.
Ostreobium, photosynthetic capacities of, 411.
Otala, permeability of epiphragm of to water vapor, 87.
Ova, Arbacia, furrow induction in, 356.
  unfertilized, of sea urchins, protein synthesis in, 209.
Ovariectomy, effect of on lizard's fat body, 325.
Oviducts of newt, sialic acid in, 266.
Oxygen consumption of endozoic algae, 411.
  perception by Callianassa, 261.
  tension in mantle cavity of Modiolus, 60.

P

P-32 uptake by holothurian, 367.
Pacemakers in tunicate heart, 434.
Palaemon, osmoregulation in, 235.
Paracalanus, effect of xanthene dyes on phototaxis of, 398.
Parasite of horseshoe crab, life-cycle of, 332.
  larva, effects of on host pituitary and gonads, 15.
Parthenogenesis in Daphnia, as affected by photoperiod, 176.
Patterns, banding, in Haliotis, 139.
Patton, W. K. Feeding habits, behavior and host specificity of Caprella, 148.
Paulson, T. C., and R. S. Scheltema. Selective feeding on algal cells by the veliger larvae of Nassarius, 481.
Pelagic larvae of Spio, 503.
Penaeus, factors affecting emergence of from substrate, 48.
Penetration of light to endozoic algae of Favia, 411.
Perception of external oxygen by Callianassa, 261.
Phaeodactylum as food for Nassarius veligers, 481.
Permeability of snail epiphragm to water vapor, 87.
Peromone-like dispersant affecting distribution of Acheta, 490.
Phosphate concentration, effect of on cell division in diatom, 199.
Photoperiod control of winter diapause in Daphnia, 176.
  relations in Ameiva, 325.
Photosynthetic capacities of endozoic algae of Favia, 411.
Phototactic behavior of Asterias, 516.
Phototaxis of copedods, influence of xanthene dyes on, 398.
Physiology, cardiovascular, of tunicates, 434.
Phytoecdysones, action of on cynthia silkworm pupae, 344.
Phytoplankton as food for Nassarius veligers, 481.
Piatigorsky, J. See A. Tyler, 209.
Pickford, G. E. See B. Lofts, 74.
Pigment in endozoic algae of Favia, 411.
  responses of Uca, comparative study of, 221.
Pigmentation of Haliotis, 139.
Pink shrimp, factors affecting emergence of from substrate, 48.
Pituitary of host, effects of cestode larva on, 15.
Plankton, pelagic larvae of Spio in, 503.
Plerocercoid larva of Ligula, effects of on pituitary and gonads of host, 15.
Poecilogony in Spio, 503.
Polychaete, morphology, feeding, and behavior of, 272.
  pelagic larvae of, 503.
  responses of to osmotic stress, 118.
  coelomic fluid and body wall, amino acids in, 35.
Polychaetes, effect of osmotic dilution on free amino acids of, 252.
Polysomes in unfertilized sea urchin eggs, 209.
Population density, role of in chemotaxis of slime molds, 298.
Preferences in feeding of Nassarius veligers, 481.
Pressure-centrifugation of Arbacia eggs, 356.
Proecdysial growth and developmental processes in crayfish, 160.
Protein synthesis in unfertilized sea urchin eggs, 209.
Provasoli, L. See A. S. d'Agostino, 1.
Pseudophyllidean cestode larva, effects of on host pituitary and gonads, 15.

Pupae of cynthia silkworm, action of ecdysone and analogues on, 344.
Puromycin, effects of on furrow induction in Arbacia eggs, 356.
  on protein synthesis in unfertilized sea urchin eggs, 209.
Pyronine, effect of on copepod phototaxis, 398.
Pyrrhocoris, use of for assay of insect hormone-like material, 154.

R

Radial organization in regenerating Tubularia, 245.
Radiophosphorus, uptake of by holothurians, 367.
Rates of cell division in diatom, effects of phosphate concentration on, 199.
Receptors, photosensitive, in Asterias, 516.
Recovery of mice following x-irradiation, 320.
Refractile bodies in Childia sperm, 382.
Regeneration in hydra, 465.
  in Tubularia, 245.
Regional differences, body, in uptake of P-32 by holothurian, 367.
Regression of testes in hypophysectomized Fundulus, effect of cold and cortisol on, 74.
Relationship between gonads and fat bodies in lizards, 325.
Reproductive cycles in lizards, relation of to fat body size, 325.
Reptile, fat body-gonadal relationship in, 325.
Respiration of endozoic algae, 411.
Responses of Nereis to osmotic stress, 118.
  of Siphonaria to osmotic stress, 96.
  of Uca chromatophores to light and temperature, 221.
Reversal of cell layers in hydra, 465.
Rhodamine, effect of on phototaxis of copepods, 398.
Ribbed mussel, air-gaping by, 60.
Roentgen-irradiation of mice, 320.
RUGH, R., AND C. SOMOGYI. Hematological recovery of mice following fetal x-irradiation, 320.
Rutilus parasite, effects of on host pituitary and gonads, 15.

S

Salamander, sialic acid in egg capsules and oviducts of, 266.
Salinity, effects of on Spio larvae, 503.
  relations of caridean shrimp, 235.
    of Nereis, 118.
    of polychaetes, 252.
    of Siphonaria, 96.
Samia pupae, assay of ecdysone and analogues on, 344.
SCHELTEMA, R. S. See T. C. PAULSON, 481.
Sea cucumber, P-32 uptake by, 367.
Sea urchin egg glycoproteins, 425.
  eggs, furrow induction in, 356.
    unfertilized, protein synthesis by, 209.
Selective feeding on algae by Nassarius veligers, 481.
Sensory perception of oxygen by Callianassa, 261.
Setae, development of in crayfish, 160.
SEXTON, O. J., AND E. H. HESS. A pheromone-like dispersant affecting the local distribution of the European house cricket, Acheta, 490.
Sexual regression in hypophysectomized Fundulus, effect of cold and cortisol on, 74.
  reproduction in Daphnia, as affected by photoperiod, 176.
Shading, reaction of Asterias to, 516.
Shrimp, external oxygen perception by, 261.
  factors affecting emergence of from substrate, 48.
Shrimps, caridean, osmoregulation in, 235.
Sialic acid in newt egg capsules and oviducts, 266.
Significance of refractile bodies in flatworm sperm, 382.
Silkworm pupae, action of ecdysone and analogues on, 344.
SIMON, J. L. Occurrence of pelagic larvae in Spio, 503.
Siphonaria, responses of to osmotic stress, 96.
SLÁMA, K., M. SUCHÝ AND F. ŠORM. Natural and synthetic materials with insect hormone activity, 154.
Slime molds, chemotaxis in, 298.
SMITH, R. E. Experimental evidence for a gonadal-fat body relationship in two teiid lizards, 325.
Snails, permeability of epiphragm of, to water, 87.
SOMOGYI, C. See R. RUGH, 320.
ŠORM, F. See K. SLÁMA, 154.
Spawning of Spio, 503.
Specificity, host, of commensal, 148.
Spermatozoa of Childia, refractile bodies in, 382.
Spio, pelagic larvae of, 503.
Spleen graft in chick embryo, growth stimulation of, 473.
Sporocyst of Microphallus, 332.
Starfish, phototactic behavior of, 516.
  commensal, feeding, behavior, and specificity of, 148.
STEVENSON, J. R., R. H. GUCKERT AND J. D. COHEN. Lack of correlation of some proecdysial growth and developmental processes in the crayfish, 160.

Stimulation of growth in chick spleen grafts, 473.
Stolon of hydroid, linear growth in, 26.
Stress, osmotic, responses of Nereis to, 118.
  of Siphonaria to, 96.
STROSS, R. G., AND J. C. HILL. Photoperiod control of winter diapause in the fresh-water crustacean Daphnia, 176.
STUNKARD, H. W. The asexual generations, life-cycle and systematic relations of Microphallus, 332.
Substrate, factors affecting emergence of Penaeus from, 48.
Succession, botanical and animal, as indicated by banding patterns of Haliotis, 139.
SUCHÝ, M. See K. SLÁMA, 154.
Swimming of Conchoecia, 456.
Symbiodinium, photosynthetic capacities of, 411.
Syncaris, osmoregulation in, 235.
Synthesis of protein in unfertilized sea urchin eggs, 209.
Synthetic materials with insect hormone activity, 154.
Systematic relations of Microphallus, 332.

T

Tactic movements in starfish, 516.
Taeniolae of Tubularia, regeneration of, 245.
Taxonomy of Magelona, 272.
  of Microphallus, 332.
Teiid lizards, gonadal-fat body relationship in, 325.
Teleost, effect of cold and cortisol on testicular regression in, after hypophysectomy, 74.
Temperature, effect of on testicular regression in hypophysectomized Fundulus, 74.
  role of in cardiovascular physiology of tunicates, 434.
    in osmoregulation of shrimp, 235.
  responses of Uca chromatophores, 221.
Tenebrio, use of for assay of insect hormone-like materials, 154.
Tentacle reorganization in Tubularia regeneration, 245.
Terrestrial snails, permeability of epiphragm of, to water, 87.
Territory formation in cellular slime molds, 298.
Testicular regression in hypophysectomized Fundulus, effect of temperature and cortisol on, 74.
Testosterone, effect of on lizard fat body, 325.
Thelepus, amino acids in coelomic fluids and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Thermoregulatory behavior in Ameiva, 325.
Thiol agent, effects of on furrow induction in Arbacia eggs, 356.
THOMAS, W. H., AND A. N. DODSON. Effects of phosphate concentration on cell division rates and yield of a tropical oceanic diatom, 199.
Tissue distribution of P-32 in holothurian, 367.
Travisia, amino acids in coelomic fluid and body wall of, 35.
  effect of osmotic dilution on free amino acids of, 252.
Trematode, life-cycle and systematics of, 332.
Triturus, sialic acid in oviducts and egg capsules of, 266.
Tropical oceanic diatom, effects of phosphate concentration on yield of, 199.
  species of Uca, comparative study of chromatophores of, 221.
Tubularia, regeneration of, 245.
Tunicates, cardiovascular physiology of, 434.
Turbellarian spermatozoa, refractile bodies in, 382.
TYLER, A., B. S. TYLER AND J. PIATIGORSKY. Protein synthesis by unfertilized eggs of sea urchins, 209.

U

Uca chromatophores, comparative study of responses of, 221.
Unfertilized sea urchin eggs, protein synthesis in, 209.
Uptake of P-32 by holothurian, 367.
Urine osmotic pressure of caridean shrimp, 235.

V

Veligers of Nassarius, selective feeding of, on algae, 481.
Ventilative behavior of Callianassa, 261.
Vertical migration of copepods, effect of xanthene dyes on, 398.
Vitellization of ovarian follicles in lizards, 325.

W

Water balance of caridean shrimps, 235.
  relations of Nereis, 118.
    of polychaetes, 252.
  vapor, permeability of snail epiphragm to, 87.
WILBUR, K. M. See T. K. S. BJÖRNBERG, 398.
WILLIAMS, C. M. Ecdysone and ecdysone-analogues: Their assay and action on diapausing pupae of the cynthia silkworm, 344.
Winter diapause in Daphnia, photoperiod control of, 176.
WORKMAN, W. M. See A. A. HUMPHRIES, JR., 266.

X

Xanthene dyes, effect of on copepod migration and phototaxis, 398.

X-irradiated mice, hematological recovery of, 320.

Y

Yield of tropical diatom, effect of phosphate concentration on, 199.

YOSHIDA, M., AND H. OHTSUKI. The photosensitive behavior of the starfish Asterias, 516.

Z

ZIMMERMAN, S. B., T. H. MURAKAMI AND A. M. ZIMMERMAN. The effects of selected chemical agents on furrow induction in the eggs of Arbacia, 356.

Zooxanthellae, photosynthetic capacities of, 411.

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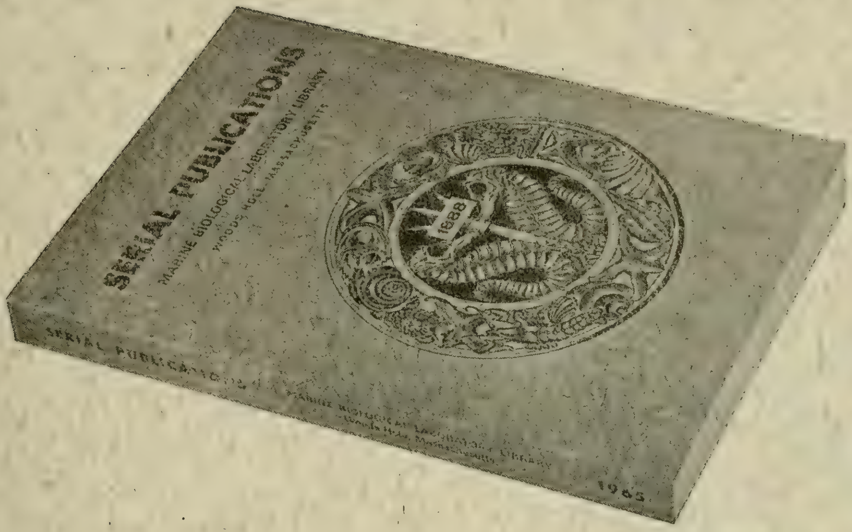
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CONTENTS

Page

AHEARN, GREGORY A.
A comparative study of P^{32} uptake by whole animals and isolated body regions of the sea cucumber Holothuria atra 367

HENLEY, CATHERINE
Refractile bodies in the developing and mature spermatozoa of Childia groenlandica (Turbellaria: Acoela) and their possible significance 382

BJÖRNBERG, T. K. S., AND KARL M. WILBUR
Copepod phototaxis and vertical migration influenced by xanthene dyes 398

HALLDAL, PER
Photosynthetic capacities and photosynthetic action spectra of endozoic algae of the massive coral Favia 411

ISHIHARA, K.
Chemical analysis of glycoproteins in the egg surface of the sea urchin Arbacia punctulata 425

KRIEBEL, MAHLON E.
Studies on cardiovascular physiology of tunicates 434

LOCHHEAD, JOHN H.
The feeding and swimming of Conchoecia (Crustacea, Ostracoda) . . 456

MACKLIN, MARTIN
Reversal of cell layers in hydra: A critical re-appraisal 465

MUN, A. M., AND JANE LONGO
Growth stimulation of embryonic spleen graft by adult chicken spleen across a membrane filter barrier 473

PAULSON, THEODORA C., AND RUDOLF S. SCHELTEMA
Selective feeding on algal cells by the veliger larvae of Nassarius obsoletus (Gastropoda, Prosobranchia) 481

SEXTON, OWEN J., AND ECKHARD H. HESS
A pheromone-like dispersant affecting the local distribution of the European house cricket, Acheta domestica 490

SIMON, JOSEPH L.
Occurrence of pelagic larvae in Spio setosa Verrill, 1873 (Polychaeta: Spionidae) 503

YOSHIDA, M., AND H. OHTSUKI
The phototactic behavior of the starfish, Asterias amurensis Lütken . 513

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CONTENTS

No. 1. August, 1968

Page

Annual Report of the Marine Biological Laboratory 1

Arme, C., and C. P. Read
Studies on membrane transport. II. The absorption of acetate and butyrate by Hymenolepis diminuta (Cestoda) 80

Bennett, Miriam F., and Judith A. Harbottle
The effects of hydrocortisone on the blood of tadpoles and frogs, Rana catesbeiana 92

Campbell, Richard D.
Colony growth and pattern in the two-tentacled hydroid, Proboscidactyla flavicirrata 96

Cheney, Daniel P.
Red cell morphometrics and volume in the cichlid teleost Tilapia mossambica, using a chromium-51 labeling method 105

Gore, Robert H.
The larval development of the commensal crab Polyonyx gibbesi Haig, 1956 (Crustacea: Decapoda) 111

Haschemeyer, Audrey E. V.
Compensation of liver protein synthesis in temperature-acclimated toadfish, Opsanus tau 130

Jeffrey, S. W.
Pigment composition of Siphonales algae in the brain coral Favia 141

Jeffrey, S. W., and F. T. Haxo
Photosynthetic pigments of symbiotic dinoflagellates (zooxanthellae) from corals and clams 149

Kriebel, Mahlon E.
Pacemaker properties of tunicate heart cells 166

Kriebel, Mahlon E.
Cholinoceptive and adrenoceptive properties of the tunicate heart pacemaker 174

Loftfield, R. B., E. A. Eigner and J. Nobel
Inter-phylogenetic specificity in the bonding of amino acids to tRNA 181

Marsh, Julian B.
Isolation and composition of a low density lipoprotein from the eggs of Arbacia punctulata 193

Niu, M. C., and A. Leikola
The developmental potentiality of the liver-RNA-treated posterior primitive streak in the chick embryo 200

PAGE

POTSWALD, HERBERT E.
The biology of fertilization and brood protection in Spirorbis (Laeospira) morchi 208

RIGBY, B. J.
Temperature relationships of poikilotherms and the melting temperature of molecular collagen 223

SHOOP, C. ROBERT
Migratory orientation of Ambystoma maculatum: Movements near breeding ponds and displacements of migrating individuals 230

UMMINGER, BRUCE L.
Polarotaxis in copepods. I. An endogenous rhythm in polarotaxis in Cyclops vernalis and its relation to vertical migration 239

UMMINGER, BRUCE L.
Polarotaxis in copepods. II. The ultrastructural basis and ecological significance of polarized light sensitivity in copepods 252

No. 2. OCTOBER, 1968

CHUANG, SHOU-HWA
The larvae of a discinid (Inarticulata, Brachiopoda) 263

HINSCH, GERTRUDE W.
Reproductive behavior in the spider crab, Libinia emarginata (L.) 273

KELLY, MAHLON G.
The occurrence of dinoflagellate luminescence at Woods Hole 279

LAMBERT, GRETCHEN
The general ecology and growth of a solitary ascidian, Corella willmeriana 296

MCPHERSON, B. F.
Feeding and oxygen uptake of the tropical sea urchin Eucidaris tribuloides (Lamarck) 308

OHTAKI, TETSUYA, ROGER D. MILKMAN AND CARROLL M. WILLIAMS
Dynamics of ecdysone secretion and action in the fleshfly Sarcophaga peregrina 322

PETERSON, MARGARET K., AND JOHN BUCK
Light organ fine structure in certain Asiatic fireflies 335

ROPES, JOHN W.
Reproductive cycle of the surf clam, Spisula solidissima, in offshore New Jersey 349

SUMMERS, WILLIAM C.
The growth and size distribution of current year class Loligo pealei 366

YONGE, C. M.
Form and habit in species of Malleus (including the "hammer oysters") with comparative observations on Isognomon isognomon 378

Abstracts of papers presented at the Marine Biological Laboratory 406

No. 3. DECEMBER, 1968

AKEY, DAVID HAROLD, AND JACK COLVARD JONES
Sexual responses of adult male Aedes aegypti using the forced-copulation technique 445

BERNS, MICHAEL W., AND WILLIAM T. KEETON PAGE
Effects of semi-starvation on growth and morphogenesis during the larval stages of a common milliped, Narceus annularis (Raf.) 454
BLUMENFELD, MARTIN, AND HOWARD A. SCHNEIDERMAN
Effect of juvenile hormone on the synthesis and accumulation of a sex-limited blood protein in the Polyphemus silkmoth 466
CASE, JAMES, AND M. S. TRINKLE
Light-inhibition of flashing in the firefly Photuris missouriensis 476
COPELAND, D. EUGENE
Fine structure of the carbon monoxide secreting tissue in the float of Portuguese man-of-war (Physalia physalis L.) 486
FREEMAN, SALLIE B.
A study of the jelly envelopes surrounding the egg of the amphibian, Xenopus laevis 501
JONES, IRA, AND THOMAS E. ROGERS
Studies on the endocommensal ciliate fauna of Caribbean sea urchins 514
MARKS, E. P., J. P. REINECKE AND R. A. LEOPOLD
Regenerating tissues from the cockroach, Leucophaea maderae: Nerve regeneration in vitro 520
OLLA, BORI L., AND WARREN W. MARCHIONI
Rhythmic movements of cones in the retina of bluefish, Pomatomus saltatrix, held in constant darkness 530
READ, LAWRENCE J.
Urea and trimethylamine oxide levels in elasmobranch embryos 537
RUSSELL-HUNTER, W. D., AND MYRA RUSSELL-HUNTER
Pedal expansion in the naticid snails. I. Introduction and weighing experiments 548
RUSSELL-HUNTER, W. D., AND MARTYN L. APLEY
Pedal expansion in the naticid snails. II. Labelling experiments using inulin 563
TELFORD, MALCOLM
The identification and measurement of sugars in the blood of three species of Atlantic crabs 574

Volume 135 Number 1

THE BIOLOGICAL BULLETIN

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The Biological Bulletin accepts papers on a variety of subjects of biological interest. In general, however, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within three months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. A *condensed title* or running head of no more than 35 letters and spaces should be included.

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THE BIOLOGICAL BULLETIN

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Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

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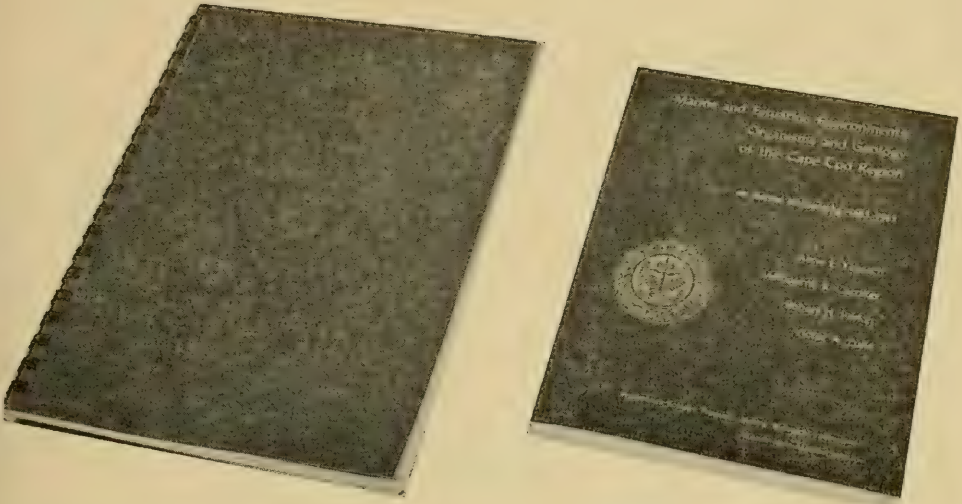
RECENT PUBLICATIONS OF THE SYSTEMATICS-ECOLOGY PROGRAM, MARINE BIOLOGICAL LABORATORY



The indexed bibliography contains a 21 page illustrated introduction to the Cape Cod Region, and is intended to introduce scientists to major papers on the geology, ecology, behavior, and related literature of the Region, and to provide the beginning for more intensive literature searches. 208 p. 1966. $5.00.

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THE
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THE MARINE BIOLOGICAL LABORATORY

SEVENTIETH REPORT, FOR THE YEAR 1967—EIGHTIETH YEAR

I. TRUSTEES AND EXECUTIVE COMMITTEE (AS OF AUGUST, 1968) 1
II. ACT OF INCORPORATION 4
III. BYLAWS OF THE CORPORATION 5
IV. REPORT OF THE DIRECTOR 7

Addenda:

1. Memorials 12
2. The Staff 24
3. Investigators, Lalor and Grass Fellows, and Students 25
4. Fellowships and Scholarships 39
5. Training Program 40
6. Tabular View of Attendance, 1963–1967 41
7. Institutions Represented 41
8. Evening Lectures 43
9. Evening Seminars 44
10. Members of the Corporation 46
V. REPORT OF THE LIBRARIAN 71
VI. REPORT OF THE TREASURER 72

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 12, 1966)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio,* who shall be the Chairman, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti,* who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEES' MEETINGS

EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members including the *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967)

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice President. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966)

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or law. (August 16, 1963)

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963)

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nomination of Officers," responsible for making nominations at the Annual Meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

I submit herewith the report of the 80th Session of the Marine Biological Laboratory. This was an eventful session. In terms of scientific enthusiasm, the period was marked by accomplishment and innovation. In a more mundane but nevertheless important sense, the final stages of our building program were funded; our endowment was substantially increased and stabilized by the sale of our General Biological Supply House holdings, and a drive has been launched among our numerous friends to complete the refurbishing of our central campus and the equipping of our new buildings.

The Marine Biological Laboratory is owned and operated by scientists. In terms of corporate structure, this may not be a unique design but in terms of actual operations, our structure *is* unique. All of the developments reported for this year, as well as past years, have been the result of cooperative and diligent efforts on the part of many individuals acting in the best interests of a service institution which is theirs in fact as well as in corporate structure. No major decision is made by any single individual or small group; all actions are within the context of policies discussed and acted upon by many. This special aspect of our progress, invaluable and unique, is one of our greatest assets and, at the same time, one of our least understood features. The support we have received during the past years is evidence that our role in science is tangibly appreciated and makes it of critical importance that we, as an institution, continue to serve the best interests of scientists, not of institutions, programs or organizations.

1. Facilities Development

Construction is now under way on the new training building and will be ready for occupancy next season. The dining-dormitory complex is fully planned, but the timing depends upon obtaining the proper building permits. These buildings are designed to satisfy, in large part, recommendations of several of our committees, especially the Buildings and Grounds Committee and the Instruction Committee. The housing of courses in the old wooden buildings provided an historical charm but lacked much in terms of stability and safety for the tools of modern research instruction. While students can survive and even thrive in cramped and antiquated living quarters, both safety and health demanded new buildings. Our mess hall has been uniformly deprecated as a physical structure for years. The new buildings will provide adequate quarters for all these purposes. In addition, they will allow us to take care of the increasing demand for year-round facilities. The new buildings represent a culmination of our plans and hopes. In this connection I would remind you of the outstanding report given by Dr. Armstrong, Chairman of the Building Committee, last year.

The major sources of funds have been The Ford Foundation and the National Science Foundation. Our gratitude to them can best be expressed by the enhanced service of the MBL to science.

2. *The Users of the MBL*

Last year approximately 270 established Ph.D. scientists used our facilities, laboratories and library for periods of two months or longer. The peak period of August found 276 in residence, the low period of December, 20 in residence. It is impossible to keep account of the numerous individuals who drop in and out, registered or unregistered, to collect specimens, read in the library or do the occasional critical experiment. The variegated and fluctuating uses of our facilities go smoothly—a remarkable tribute to the genius of our operating personnel.

A word should be added about the composition of our scientist clientele. Over the years, there has been a yearly turnover of about 30%. That is, 30% of those in residence in a given year were not in residence the year before. This is good; MBL must not bcome a private club. Our facilities must be allocated on the basis of intellectual need. Membership in the corporation signifies support of the institution, not a vested interest in its services.

3. *The Woods Hole Scientific Community*

Excluding tourists, the largest single population component of Woods Hole is composed of individuals present in the area for scientific reasons. The organizations providing the physical base for the scientific population have a common general objective of forwarding scientific inquiry; the devices used towards furthering this are varied. Scientists cooperate when there is intellectual reason to do so. Organizations may co-exist in a sort of mutualism, promoting scientific cooperation while studiously avoiding interference with scientists by administrative rigidity or exclusiveness. Woods Hole does enjoy a community of scientists and the organizations do enjoy a mutualism. The Woods Hole Oceanographic Institution, with its productive staff and programs, provides a worldwide oceanic oversight second to none and the Bureau of Commercial Fisheries Laboratory, as an integral part of the federal establishment, furnishes the expertise in applied aspects of exploitation of the oceans. The Marine Biological Laboratory, as the largest and best known service laboratory for biology in the world, provides an intellectual framework that could never be achieved by a single programmatic institution such as a university. Our faculty, to use the term in a very broad sense, is truly worldwide and its influence cannot be measured in terms of time, or dollars, or numbers, but only in terms of intellectual achievement.

I am happy to state that scientific cooperation and administrative co-existence in Woods Hole are in a productive if not utopian state. Ideas are exchanged, facilities are shared, and people talk to each other. Any formal inter-institutional arrangements within the Woods Hole community are certainly not to be sought; each institution has its unique way of doing things. I am personally proud to have a multiple set of duties, as an individual but not as an administrative siamese twin.

4. How Large is Too Large

In 1937 during August the MBL provided services for 256 senior investigators. This required 73,000 square feet of space, not counting course facilities. In 1967, thirty years later, the peak of 276 senior investigators in residence required space plus supporting facilities of 122,000 square feet. The demand for space increases each year, not rapidly but appreciably. This raises the question: How large should MBL be in terms of peak number of senior investigators? The Executive Committee and Trustees have adopted a very cautious attitude towards the size of MBL. Recognizing that a major contribution of MBL is to provide facilities for scientific work, they see that exchange of ideas and information is equally if not more responsible for the global impact of MBL on the advancement of science. The growth of ideas may well be a more important contribution of scientists working at MBL than is the production of new results. So far, I think we all come out with the idea that MBL is about large enough in terms of numbers of people. The physical size of our plant, however, must continue to grow to serve the stable population size. Our library holdings show a doubling time of less than ten years. This means that ten years from now much more of the Lillie Building will be occupied by the Library. Similar reasoning shows increased needs for physical space for the machines and materials of modern science. I do not think we are too large in terms of number of production scientists but I suspect we are large enough. But unless we anticipate a reduction in our intellectual size, we must continue to grow in terms of our physical plant.

5. Attendance—1967

Please refer to the tabular report of attendance.

A precise head count is difficult since there is a considerable number of individuals using our facilities throughout the year for library work, short-term collecting, and consultation. While we know who is here at any one time, no consistent record is maintained for short-term visitors.

From the number of inquiries, it is clear that much more extended use of our facilities over a broader yearly time scale will follow the completion of our new dining-dormitory complex. Figures for attendance for those duly registered are given in the tabular reports.

6. The Fifth Anniversary of the Systematics-Ecology Program

Founded in 1962, the Systematics-Ecology Program is a going concern. Under the stewardship of Dr. Carriker, boats, trucks, collections, and other items of logistic support are now provided for investigations into ecology, biogeography and systematics of the Cape Cod area. The program has placed MBL in a position to offer to scientists who wish to study organisms in natural habitats the same fine services as we have for many years offered to those working more specifically with marine forms as experimental material.

In addition, and equally important, have been the direct contributions to science made by individuals working with SEP. Some three dozen senior investigators (*i.e.,* doctoral-plus level) have carried out studies. Ten pre-doctoral students have been trained. Over 150 reports have been made, 28 as major papers in established journals, the rest as reports to scientific meetings and other items existing as abstracts. A manual on preservation of marine forms, a key in invertebrate forms, and a bibliography of marine and estuarine environments have been published and are available at cost from the Supply Department. Also, the Gray Museum is assuming a position of real importance, preserved specimens being available for ready identification of experimental material. A most valuable feature is the collection of colored slides.

These accomplishments in so short a time are impressive: the ground work has been laid to allow MBL to be of service to a community of scientists that might not have been so interested in our establishment in the past. Dr. Carriker has made good progress towards the objectives of SEP. The most cogent way of stating these objectives is to quote from his original plan:

"It should be clear from the foregoing that the explicit intent of the Systematics-Ecology Program is to provide opportunity for individual investigators, or investigators and their graduate students, to pursue research in marine systematics and/or ecology in areas of their research interests within the overall framework of the Program, and under as ideal a research environment as can be devised. Living organisms, chemicals, general supplies, etc., would be available through the service departments of MBL at the expense of the Program. It is to be the function of the Director to provide direction to the Program, facilities, support, program ideas where desired, and to attract visiting investigators who should help decide the course of their researches."

7. *The MBL and the Oceans*

The Marine Biological Laboratory was founded for the explicit purpose of providing a good place for scientists to work with the advantages of the rich biological resources of the sea. The name and location lend emphasis to the virtues of marine forms but this has never been restrictive. During the past decade worldwide interest has been sharpened in the resources of the sea with strong attention to oceanography and fisheries. Recognition of the importance of studying the sea was early appreciated by MBL scientists. Frank Lillie chaired the National Academy of Sciences Committee, whose recommendations led to the founding of Woods Hole Oceanographic Institution and the enhancement of the Scripps Institute of Oceanography. MBL is fortunate in being located in immediate proximity to oceanographic and fisheries institutions and hopefully will long continue to maintain its pervasive role as a general biological laboratory devoted to the support of sound research directed towards the solution of basic biological problems.

A Sea Grant College Act was enacted by Congress a year ago and a program has been established in NSF. A Marine Sciences Act has established a Commission and a Council charged with recommending National Programs in Marine Sciences and Engineering. Reports have been issued by the council and should be forthcoming soon from the commission. The MBL, as a resource for basic investigations of biological problems, including use of marine forms, is clearly in a position to exercise leadership in these developments, not as an institution perhaps but because scientists working at MBL have an awareness of the value of work on marine forms that will help guide the more applied developments. I think none of us visualizes MBL as an operating unit of the Sea Grant Complex of the future, but our guidance and enthusiasm are surely needed. Our mission is primarily that of assisting basic biological research.

8. The Fund Drive

At our last meeting the Corporation authorized an effort to obtain additional funds to complete the current building program. To my knowledge this is the first time that the institution has thus formally appealed to its constituents. Records do show that, in the early days, collections were made now and then to pay to have the floors swept, but until rising costs caught up with us, MBL has been able to avoid direct organized solicitation. I am sure all will agree that the request is appropriate and I hope that the response will enable us to finish our program we have planned. After all, as a corporation chartered in the Commonwealth of Massachusetts, we could not, and would not, dip into our meagre capital reserves.

9. A Word on the Care and Feeding of Scientists

We are all aware of the success of our Cottage Colony. This is our major bulwark to insure that use of MBL for research is not limited to those established individuals who own property in the area or who can afford to rent living quarters on the open market. The Cottage Colony exists to take care of the housing needs of young investigators with families and of visitors with families from abroad. Our cottages are nice places to live in and therein lies a danger. Continued occupancy by a single family unit over a period of many years would be contrary to the purposes for which the cottages were provided by foundation and private gifts. It is essential that cottages, apartments, and dormitory rooms never become part of a vested interest.

MBL attempts to provide food services. There has been healthy criticism of these services over many decades. Meanwhile there have been no cases of malnutrition nor epidemics. A highly competent committee is now functioning to make recommendations about our prandial future—nutritional, aesthetic and social.

10. Gifts and Bequests

We continue to take pride in the support afforded by our friends in the community through the MBL Associates. The Laboratory was saddened by the death of Mrs. G. H. A. Clowes, who for many years gave much of herself to us. It was most heartening that her daughter-in-law, Peggy—Mrs. G. H. A. Clowes, Jr.—was able to succeed her as president of the Associates. Our small Supply Department fleet will be graced this year with the addition of the 33′ "Dasya" the gift of Mr. Charles L. Morse, Jr. of Penzance Point, his former "Chadroe."

11. Personnel

Finally my thanks and appreciation to our resident staff who provide the important services required throughout the laboratory. It is with sorrow I report the death of Mr. Robert Adams in March, so soon after his retirement at the end of the year.

1. Memorials

ALEXANDER FORBES

By Alfred C. Redfield

Alexander Forbes died on March 27, 1965, at Milton, Massachusetts, where he had lived since his birth eighty-three years before. He was of the tradition of those nineteenth century men of independent means who in following their curiosity about the phenomena of nature added much to the advancement of science.

His major accomplishments were in neurophysiology, a career he had been encouraged to enter by G. H. Parker and Walter Cannon. Visiting England as a young man he brought to this country first-hand knowledge of the work of Sherrington on reflex action and of Lucas and Adrian on the nature of nervous conduction. Much of his subsequent effort was an attempt to understand the former in terms of the latter. For a period his laboratory at the Harvard Medical School became the leading center in America for neurophysiological investigations. He was the first to apply vacuum tubes to the amplification of bioelectric currents and thus became one of the progenitors of the ubiquitous black boxes of the modern laboratory.

Alex Forbes' energies were too great to be exhausted by a distinguished career in science. His keen sense of social responsibility led him to give unstintingly of his time and means to good causes in which he believed. For many years he was President of the Board of Trustees of the George Junior Republic. He encouraged and supported many young people whose ambitions appealed to him, frequently through channels unknown to them. His avocations were yachting, aviation, and outdoor sports, such as mountain climbing, and skiing—pleaures he could share with others. He was never happier than when having a merry time with a group of friends.

Stimulated by Sir William Grenfell, his combined interests in boating, aviation, and his concern with the welfare of those who follow the sea led him to organize and carry out in 1931 an aerial survey of the coast and mountains of northern Labrador. For this accomplishment he was awarded the Daly Medal of the American Geographical Society. Labrador led to his assignment during the Second World War to lay out an air route across this desolate northern country.

Alexander Forbes became a member of the Corporation of the Marine Biological Laboratory in 1925 and a Trustee of the Woods Hole Oceanographic Institution in 1937. He published two papers on work done at the Laboratory. One was on the avicular organ of *Bugula,* the other on the viscosity of the *Nereis* egg as influenced by radiation. These papers and others on flying fish and the flight of gulls are evidence of the diversity of his interests in biology. His understanding of our purposes and his friendships with many of us made him our natural ambassador to Naushon Island which was his base of operation each summer. Laboratory people have always been generously welcome on the Island and many of us recall with gratitude his personal hospitality, both at Naushon and on his yachts.

The Alexander Forbes Lectureship was established in 1959 by the Grass Foundation, of which the majority of Trustees were at some time his laboratory associates. It provides a lasting memorial to an honored and beloved friend of the Marine Biological Laboratory.

2. The Staff

EMBRYOLOGY

I. Instructors

Malcolm S. Steinberg, Professor of Biology, Princeton University, in charge of course
James N. Cather, Associate Professor of Zoology, University of Michigan
Ralph Hinegardner, Assistant Professor of Biology, Columbia University
Irwin R. Konigsberg, Professor of Biology, University of Virginia
James W. Lash, Associate Professor of Anatomy, University of Pennsylvania
Hans Laufer, Associate Professor of Zoology, University of Connecticut

II. Junior Instructors

Max Burger, Assistant Professor of Biology, Princeton University
Gary Freeman, Assistant Professor of Zoology, University of Illinois

III. Laboratory Assistants

Burr G. Atkinson, Jr., University of Connecticut
William F. Sindelar, Western Reserve University

IV. Lectures

M. S. Steinberg	Introduction to the course
I. R. Konigsberg	Teleosts I
I. R. Konigsberg	Teleosts II
M. S. Steinberg	Coelenterates I
M. S. Steinberg	Coelenterates II
Dorothy Spangenberg	Initiation of metamorphosis (strobilization) in *Aurelia*
Fred Diehl	Regeneration in Hydroids
G. Freeman	Ctenophores I
G. Freeman	Ctenophores II
G. Freeman	Sponges
C. R. Austin	Fertilization
Max Burger	Biosynthesis of structural elements of cell walls and membranes
D. L. D. Casper	Symmetry and self-assembly
Hugh Huxley	Self-assembly of the contractile units of muscle
M. S. Steinberg	Self-assembly of multicellular complexes
Robert Rosen	Pattern generation in some model embryological systems
Tom Humphreys	A biochemical approach to species-specific aggregation in marine sponges
Lionel Jaffe	The early development of *Fucus:* Prototype of localization
Everett Anderson	The fine structure of eggs
Raymond Rappaport	Cytokinesis
Paul Green	Biochemistry and physics of cell morphogenesis
Round Table Discussion	What is differentiation?
J. Cather	Spiralians I
J. Cather	Spiralians II
J. Cather	Spiralians III
Jack Collier	Biochemistry of the *Ilyanassa* embryo
John M. Arnold	Development of cephalopods

James Lash	Ascidians I
James Lash	Ascidians II
James Lash	Ascidians III
Roger Milkman	The genetics and development of *Botryllus*
H. Laufer	Crustaceans I
H. Laufer	Crustaceans II
Philip Siekevitz	The assembly of cellular membranes
H. Laufer	Crustaceans III
R. Hinegardner	Echinoderms I
R. Hinegardner	Echinoderms II
John Hughes	The sex life of the lobster
Paul Weisz	The significance of larvae
Round Table Discussion	Unsolved problems in development
Edward Kellenberger	Shape-inheritance of a protein polymer (Morphogenesis of phage T4 capsid)
Michail Fischberg	Germinal cytoplasm in gall midges and anurans
Helge Stalsberg	Morphogenetic movements and differential mitotic activity during early heart formation in the chick embryo
James Lash	Chondrogenesis: Genotypic and phenotypic expression
Howard Green	Cell contact and oncogenic viruses
Allison Burnett	Dedifferentiation, metaplasia, and ions

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania

Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory

W. D. McElroy, Director, McCollum-Pratt Institute, The Johns Hopkins University

J. Woodland Hastings, Professor of Biology, Harvard University

II. Instructors

Andrew G. Szent-Györgyi, Professor of Biology, Brandeis University, in charge of course

Sydney Brenner, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England

Roderick K. Clayton, Professor of Biophysics, Cornell University

Harlyn Halvorson, Professor of Molecular Biology and Bacteriology, University of Wisconsin

Hugh E. Huxley, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England

Maurice Sussman, Professor of Biology, Brandeis University

K. E. Van Holde, Professor of Physical Chemistry, University of Illinois

III. Staff Associates

Henry DePhillips, Trinity College

George Hoch, University of Rochester

Eugene Katz, Laboratory of Molecular Biology, Cambridge, England

David Patterson, Brandeis University

Michael Smollen, University of Rochester

William Steinberg, University of Wisconsin
Raymond E. Stephens, Harvard University
James C. Vary, University of Wisconsin
Anne Marie Weber, St. Louis University
Bernard Weissblum, University of Wisconsin

IV. Special Lecturers

Shinya Inoué, Professor of Biology, University of Pennsylvania
John Nicholls, Associate Professor of Physiology, Yale Medical School
Sol Spiegelman, Professor of Microbiology, University of Illinois

V. Assistants

Robert Levy, New York University
Patricia S. Vary, University of Wisconsin

VI. Lectures

Andrew G. Szent-Györgyi	Muscle contraction—I
	Muscle contraction—II
Anne Marie Weber	Control of contraction
K. E. Van Holde	Protein structure—I. Determinants of secondary and tertiary structure
	Protein structure—II. Quaternary structure
	Protein structure—III. Control
H. O. Halvorson	Developmental changes during sporulation: Physiological and genetic control of the formation of the dormant state
William Steinberg	Developmental changes during outgrowth: Protein synthesis and ordered enzyme synthesis
H. O. Halvorson	Developmental changes during the cell cycle: Effect of gene position on enzyme timing in yeast
Maurice Sussman	I—Some conceptual and operational aspects of developmental biochemistry
	II—The cellular slime molds—LCVII
	III—The cellular slime molds—LCVIII
Roderick K. Clayton	The mechanism of photosynthesis:
	I—Early formulations and the question of quantum efficiency
	II—Contemporary formulations and the cooperation of two photochemical processes
George Hoch	III—Oxido-reduction and phosphate esterification
Roderick K. Clayton	IV—Physical problems and mechanisms
Hugh E. Huxley	Muscle structure—I
Bernard Weissblum	The role of tRNA in the synthesis of various non-proteins
Hugh E. Huxley	Muscle structure—II
Sydney Brenner	The genetic aspects of the code—I
	The genetic aspects of the code—II
	The genetic aspects of the code—III
Shinya Inoué	Organization of living cell fine structure: I—The mitotic spindle
Raymond E. Stephens	II—Structural proteins associated with mitosis

R. Wolfe	Methane bacteria and transmethylation
Shinya Inoué	Organization of living cell fine structure: III—Sperm chromosomes
John G. Nicholls	Electrical signalling in the nervous system
E. Kravitz	A study of synaptic chemistry in single neurons
John G. Nicholls	Neural organization in the visual system
	Long-term changes in synaptic transmission
F. Beckwith	Regulation of gene action
B. D. Davis	Regulation of RNA and DNA synthesis
Seymour Beychok	Optical activity and conformation of ribonuclease
Cyrus Levinthal	Pathways of protein foldings
L. Lorand	Enzyme-controlled assembly of protein fibers: Biosynthesis of a blood clot
Albert Szent-Györgyi	Comments
Ruth Hubbard	Chemistry of visual excitation
Richard Cone	Early receptor potentials
William Hagins	Excitatory processes in squid eye
J. E. Dowling	Visual adaptation and visual pathway anatomy
E. F. MacNichol, Jr.	Retinal mechanisms of color discrimination and spatial contrasts
George Wald	Mechanisms of human color vision
Sol Spiegelman	*In vitro* RNA synthesis
Robert Martin	Electrical and chemical synaptic transmission in ciliary ganglion
David Yphantis	Equilibrium centrifugation of non-ideal protein solutions
A. H. Sturtevant	T. H. Morgan
Douglas R. Wilkie	A muscle physiologist's lament: Is thermodynamics useless?
E. A. Adelberg	DNA transfer in bacterial conjugation
Jean-Pierre Changeux	On the mechanism of allosteric interaction
I. C. Gunsalus	Gene transfer in the fluorescent pseudomonads and the biological management of structural diversity

MARINE BOTANY

I. Instructors

Walter R. Herndon, Professor of Botany and Assistant Vice-President, Academic Affairs, University of Tennessee, in charge of course

Philip W. Cook, Assistant Professor of Botany, University of Vermont

Melvin S. Fuller, Associate Professor of Botany, University of California, Berkeley

H. Wayne Nichols, Associate Professor of Botany, Washington University

Janet R. Stein, Associate Professor of Botany, University of British Columbia, Vancouver

II. Special Lecturers

R. W. Wilce, University of Massachusetts

L. Provasoli, Haskins Laboratories

R. Drum, University of Massachusetts

I. M. Lamb, Harvard University

F. Trainor, University of Connecticut

F. E. Round, University of Bristol, England

III. Assistants

Davis L. Findley, University of Tennessee
Diane I. Findley, University of Tennessee
Thomas Lee, University of Connecticut

IV. Lectures

W. R. Herndon	Marine environment and local flora
J. R. Stein	Introduction to algae
M. S. Fuller	Cyanophyta—I
J. R. Stein	Cyanophyta—II
M. S. Fuller	Myxomycota and Eumycota (Introduction)
P. W. Cook	Isolation and cultivation of algae and fungi
M. S. Fuller	
J. R. Stein	
P. W. Cook	Chlorophyta (Introduction)
	Work on isolation technique
W. R. Herndon	Chlorophyta—Chlorococcales
P. W. Cook	Chlorophyceae—Volvocales
W. R. Herndon	Chlorophyceae—Tetrasporales
M. S. Fuller	Free-living phycomycetes
J. R. Stein	Prasinophyceae
	Chlorophyceae—Ulotrichales I
	Chlorophyceae—Ulotrichales II
P. W. Cook	Chlorophyceae—Cladophorales, Siphonocladales, Codiales
P. W. Cook	Chlorophyceae—Codiales, Dasycladales, Derbesiales
J. R. Stein	Chlorophyceae—Oedogoniales, Zygnematales
W. R. Herndon	Euglenophyceae
Lionel Jaffe	The early development of *Fucus*
I. Freidmann	Cytology of fertilization in algae
J. R. Stein	Xanthophyceae
J. R. Stein	Chrysophyceae, Haptophyceae
M. S. Fuller	Fungal parasites of algae
P. W. Cook	Phaeophyceae—Introduction
J. R. Stein	Phaeophyceae—Ectocarpales, Sphacelariales
P. W. Cook	Phaeophyceae—Chordariales, Desmarestiales, Sporochnales
W. R. Herndon	Phaeophyceae—Punctariales, Dictyosiphonales, Dictyotales, Cutleriales
R. W. Wilce	Arctic algal ecology
M. S. Fuller	Physiology of marine fungi
J. R. Stein	Phaeophyceae—Laminariales
P. W. Cook	Phaeophyceae—Fucales
H. Wayne Nichols	Rhodophyceae—Introduction
L. Provasoli	Nutrition and culture of marine algae
H. Wayne Nichols	Rhodophyceae—Bangiophycidae
R. Drum	Ultrastructure of diatoms
H. Wayne Nichols	Rhodophyceae—Transitional forms, Nemalionales, Gelidiales
I. M. Lamb	Zonation of marine algae of Northeast Atlantic Coast
H. Wayne Nichols	Rhodophyceae—Cryptonemiales, cytology of reproduction
M. D. Fuller	Fungi

H. WAYNE NICHOLS	Rhodophyceae—Gigartinales, Rhodymeniales
H. WAYNE NICHOLS	Rhodophyceae—Ceramiales
F. TRAINOR	Morphogenesis in coenobial algae
H. WAYNE NICHOLS	Rhodophyceae—Summary evolution, experimental aspects of development
W. R. HERNDON	Charophyta
M. S. FULLER	Fungi—marine ascomycetes
M. S. FULLER	Pyrrophyta
F. E. ROUND	Bacillariophyceae I
	Bacillariophyceae II

INVERTEBRATE ZOOLOGY

I. CONSULTANTS

FRANK A. BROWN, JR., Morrison Professor of Biology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
CLARK P. READ, Professor of Biology, Rice University
ALFRED C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

W. D. RUSSELL-HUNTER, Professor of Zoology, Syracuse University, in charge of course
GEORGE G. HOLZ, JR., Professor of Microbiology, State University of New York, Upstate Medical Center, Syracuse
NORMAN MILLOTT, Professor of Zoology, Bedford College, University of London, England
ERIC L. MILLS, Associate Professor of Biology, Institute of Oceanography, Dalhousie University, Halifax, Nova Scotia, Canada
JAMES CASE, Professor of Biology, University of California, Santa Barbara
FRANK M. FISHER, JR., Assistant Professor of Biology, Rice University
ROBERT K. JOSEPHSON, Associate Professor of Biology, Western Reserve University
JONATHAN P. GREEN, Assistant Professor, Division of Biological and Medical Sciences, Brown University
MEREDITH L. JONES, Curator in charge, Division of Worms, United States National Museum, Smithsonian Institute

III. JUNIOR INSTRUCTOR

HUGH Y. ELDER, Lecturer in Histology, Institute of Physiology, University of Glasgow, Scotland

IV. ASSISTANTS

ALBERT J. BURKY, Syracuse University
DARRELL R. STOKES, University of Hawaii

V. LECTURES

W. D. RUSSELL-HUNTER	Orientation and functional homologies in invertebrates
ALBERT J. BURKY	Laboratory orientation
DARRELL R. STOKES	

Robert K. Josephson	Cnidaria I—Introduction to the Cnidaria and Ctenophora
	Cnidaria II—Nervous system and behavior
	Seminar—Physiological mechanisms controlling behavior in the hydroid, *Tubularia*
	Cnidaria III—Function of the nematocysts, feeding
Frank M. Fisher, Jr.	Turbellaria and Trematoda
	Cestoda and Rhynchocoela
W. D. Russell-Hunter	Mollusca I—General molluscan organization: Functioning of mantle cavity in Gastropoda
	Littoral Ecology: Theoretical and physiological
Eric L. Mills	Littoral Ecology: Practical and pragmatic
W. D. Russell-Hunter	Mollusca II—Gastropoda (continued): Mantle cavity and feeding mechanisms in Bivalia
	Mollusca III—Adaptations in bivalves: Aspects of general physiology of gastropods and bivalves
	Mollusca IV—Functional morphology in Cephalopoda and minor groups
	Short seminar—Some problems of mechanics in molluscs
	Mollusca V—Functional morphology in Amphineura, *Neopilina,* archetypes and ancestors
Eric L. Mills	The framework of Cape Cod—an introduction to the history of the Cape and Barnstable Harbor
Meredith L. Jones	Annelida introduction: General characteristics, classification, external morphology
	Annelida II—Feeding, respiration and osmoregulation
	Polychaetous serendipity: *Magelona* and *Caobangia*
	Annelida III—Reproduction, nervous system, locomotion
W. D. Russell-Hunter	Seminar—Physiological variation and evolution in freshwater molluscs
Eric L. Mills	Arthropoda I—General features of arthropods: Introduction to crustacean structure
	Arthropoda II—Crustacean structure, physiology, and reproduction
James Case	Arthropoda III—Neurosecretion and endocrine control
	Arthropoda IV—Vegetative physiology
W. D. Russell-Hunter (Chairman)	Symposium—Invertebrate connective tissues, skeletons and mechanics
Hugh Y. Elder	The function of connective tissues in invertebrates and the occurrence of elastic fibers
Robert V. Rice	Amino acid composition of bivalve ligaments
Elaine A. Robson	Functions of mesoglea in coelenterates
Philip Person	Endoskeletal cartilages in invertebrates
Robert K. Josephson	Tentacular rowing by sea anemones
James Case	Arthropoda V—Neurobiology
Frank M. Fisher, Jr.	Aschelminthes
	Entoprocta, Ectoprocta
Norman Millott	The biology of asteroids and ophiuroids
A. Farmanfarmaian	Function of hemal system and axial organ in echinoids
Norman Millott	Photosensitivity in echinoids
	The biology of echinoids
	The biology of holothurians; development and phylogeny of echinoderms

Eric L. Mills	Short seminar—The biology of an amphipod crustacean sibling species pair
Jonathan P. Green	Protochordata I Protochordata II Porifera
George G. Holz, Jr.	The nature of the Protozoa
Hugh Y. Elder	Body cavities, connective tissues, and invertebrate locomotion
George G. Holz, Jr.	Flagellates I Flagellates II—Sarcodines I
Jean-Marie Bassot	Structure and evolution of luminous organs in some marine animals
George G. Holz, Jr.	Sarcodines II Ciliates
W. D. Russell-Hunter	One approach to the zooplankton

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Marine Biological Laboratory
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
John H. Ryther, Woods Hole Oceanographic Institution

II. Instructors

W. Rowland Taylor, Associate Professor of Oceanography, The Johns Hopkins University, in charge of course

Dennis J. Crisp, Director, Marine Science Laboratories, University College of North Wales, U. K.

Howard L. Sanders, Senior Scientist, Woods Hole Oceanographic Institution

Lawrence B. Slobodkin, Professor of Zoology, University of Michigan

Gotram Uhlig, Biologische Anstalt Helgoland, Germany

III. Special Lecturers

Luigi Provasoli, Haskins Laboratories, New York
Victor Zullo, Systematics-Ecology Program, Marine Biological Laboratory

IV. Assistants

John F. Boyer, University of Chicago
Herman F. Bosch, The Johns Hopkins University

V. Lectures

W. Rowland Taylor	Introduction to marine ecology The marine environment I—Chemistry of sea water The marine environment II—Solar radiation through sea water Phytoplankton I Phytoplankton II

	Primary productivity by phytoplankton
	Phytoplankton III
	Ecological techniques: Salinity, dissolved oxygen, chlorophyll
	Ecological techniques: Plankton sampling gear
V. Zullo	Cape Cod as an ecological laboratory
	The evolutionary significance of the deep-sea barnacle fauna
Dr. Leadbetter	Introduction to microbial ecology
	Microorganisms and their role in nature
	Selective cultures
H. Jannasch	Microbial transformations I
	Microbial transformations II
	Continuous culture in microbial ecology
	Bacteriological lab and field techniques
	Recent approaches in microbial ecology
Dr. Truper	Ecology of photosynthetic bacteria
D. Rhodes	Environmental stress gradients and paleobathymetry
H. Bosch	Benthic collecting methods
R. Green	Population dynamics of *Gemma gemma*
M. Jones	On the spatial distribution of selected benthic invertebrates of San Francisco Bay
R. Hessler	Studies of the deep-sea benthos
J. Simon	Reproductive ecology of polychaetes
R. Scheltema	The importance of the larvae of benthic marine invertebrates
E. Uchupi	Bathymetry and sediments of the shelf and slope of the Atlantic Coast and Gulf of America
G. Uhlig	Physiological ecology—a new field of research
	Effects of temperature and salinity on marine and brackish water animals
	Introduction to benthic microfauna
	Methods of research on benthic protozoa
	The biology of folliculinids (Ciliata; Heterotrichida), illustrated with film
	Life-cycle and ecological observations on *Noctiluca miliaris,* illustrated with film
R. Wilce	Arctic ecology
L. Slobodkin	Ecology of evolution
	Classical population dynamics
	Intrinsic rates of increase and reproductive value
	Energetics of populations and the theory of predation
	Control of abundance in nature
D. Crisp	The future of ecology in practical affairs
	The role of larvae in the ecology of marine invertebrates
	Behavior of marine invertebrate larvae during the free swimming period
	Settlement behaviour as exemplified by the cyprid and other larval forms
	Behavior of the larvae of deposit living invertebrates
	Gregarious behavior at settlement
	Territorial behavior at settlement

SYSTEMATICS–ECOLOGY PROGRAM

THE STAFF

Director: MELBOURNE R. CARRIKER

Resident Systematist: VICTOR A. ZULLO

Resident Ecologist: ROGER H. GREEN

Resident Biologist, Assistant Director and Chief Diver: DAVID C. GRANT

Postdoctoral Fellows and Research Associates: MICHAEL T. GHISELIN, THOMAS J. M. SCHOPF, JOSEPH L. SIMON, WILLIAM C. SUMMERS, BARRY A. WADE, DAVID K. YOUNG

Graduate Research Trainees: WILLIAM R. COBB, WILLIAM D. HUMMON, ALLAN MICHAEL, LELAND W. POLLOCK, JAMES R. SEARS

Visiting Investigators in Residence: EDWARD L. BOUSFIELD, RALPH O. BRINKHURST, LOUISE BUSH, GARY CHARLTON, HOWARD H. CHAUNCEY, EDWIN T. MOUL, PHILIP PERSON, HAROLD H. PLOUGH, FRANK E. ROUND, ROBERT T. WILCE, DONALD J. ZINN

Consultant: WILLIAM RANDOLPH TAYLOR

Curators: PETER E. SCHWAMB, JOHANNA REINHART

Biophotographer and Field Units: PAUL A. SHAVE

Artists: RUTH VON ARX, LINDA ROGERS

Captain, R/V A. E. VERRILL: JAMES P. OSTERGARD

Administrative Assistant: HAZEL F. SANTOS

Program Secretary: EVA S. MONTIERO

Research Assistants: H. SUZANNE BOLTON, JAMES C. BOYCE, BRUCE FOUND, KATHERINE HOBSON, CAROL KOURTZ, GEORGE R. LILLY, STEPHEN LIPSON, HAROLD MARSH, JON E. MACDONALD, RICHARD A. MCGRATH, CHARLES R. MCKAY, PAULA L. MOGILNI, JUDY MURPHY, BETSY PALMER, PENNY PINTER, ARMAND ROBERGE, STUART L. SANTOS, ANNE SMARSH, JANIS A. SPEEL, DIRK VAN ZANDT, MARILYN ZEIGLER

Visitors: WILLIAM D. and MADELINE P. BURBANCK, WENDELL K. PATTON, MADELENE E. PIERCE, SAMUEL C. MOZLEY

SEMINARS (WINTER INCLUDED)

P. WEYGOLDT	Comparative reproductive biology in pseudoscorpions
JAMES R. SEARS	Developmental growth patterns and the occurrence of "mitotic waves" in the coenocytes of the marine endophytic green alga, *Blastophysa rhizopus* Reinke
DAVID K. YOUNG	The food and feeding of Indo-West-Pacific dorid nudibranches
ROGER H. GREEN	Constant *versus* erratic mortality as a population regulator in the intertidal
KENNETH J. BOSS	Studies in the systematics of the Tellinidae
TORE LEVRING	Submarine light and vertical distribution of marine benthic vegetation
GARRY CHARLTON	Determination of O_2 in biological tissues
H. PERRY JEFFRIES	Environmental stress on marine communities as revealed by fatty acid and amino acid spectra
ARTHUR C. MATHIESON	Ecological studies of the marine brown alga *Phaeostrophion irregulare* S. et G. on the Pacific coast of North America
HAROLD H. PLOUGH	The distribution of ascidian species in the Woods Hole region

Saul B. Saila	Some aspects of the movement and behavior of the lobster, *Homarus americanus*
Arthur Humes	Concepts and recent trends in work on parasitic copepods
David Dean	Maine's new marine laboratory—The Ira C. Darling Center for Research, Teaching and Service
Katharine Hobson	The feeding and ecology of two sympatric species of lugworm (Arenicolidae Polychaeta)
Alfred Senft	Schistosome physiology II—a continuation
Joseph L. Simon	The biology of *Histriobdella homari,* a commensal polychaete
Meredith L. Jones	Polychaete potpourri
William C. Summers	Local squid: past, present and future
Louise Hobson	Speculation on the transport of phytoplankton carbon into the deep sea
Thomas J. M. Schopf	Is an ectoproct possible? (Oxygen consumption in a small marine invertebrate)
Lawrence Pinter	Behavior of *Phidippus* (Salticidae, Arachnida)
Daniel Merriman	The history of oceanography
Holger W. Jannasch	Bacterial growth kinetics in microbial ecology
John D. Davis	Selective attack by the boring polychaete worm, *Polydora websteri:* environment *vs.* distribution
Robert A. Croker	The biology of some closely-related sympatric amphipods
Robert T. Wilce	Arctic algal ecology
A. Lee McAlester	Paleobiology of bivalve molluscs
Barry A. Wade	Ecology of New England nudibranchs
Harold H. Plough	Recollections of Woods Hole, 1913–1923: Problems and personalities
Victor A. Zullo	Barnacles in Europe
Ruth D. Turner	Deep-sea boring bivalves
William J. Clench	Tree snails of the genus *Liguus* in Florida, Hispaniola and Cuba
Gilbert V. Levin	The ecology of Mars
Martyn Apley	On *Melampus bidentatus,* the salt-marsh snail
Michael T. Ghiselin	The principles and concepts of systematic biology
David K. Young	Interpretations from bulk chemistry of Chesapeake Bay sediments
Gunther Stotzky	Influence of clay minerals and the ecology and population dynamics of microorganisms
Melbourne R. Carriker	The mechanism of shell penetration by muricid gastropods: chemical or mechanical?
William D. Burbanck	The ecology of *Cyathura:* The development of a problem
Paul S. Galtsoff	Water quality levels essential for the well-being of aquatic biota (summary of the work of Technical Advisory Committee of FWPCA)
K. O. Emery	Relict sediments of the continental shelf
Ralph O. Brinkhurst	Biology of aquatic oligochaetes
William T. Lammers	Biophysical limnology: The separation of colloidal and suspended particles in natural waters
Frank E. Round	Vertical migration rhythms in benthic algae
Donald J. Zinn	History and biology of Penikese Island: 1872–present

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ROBERT GUNNING, Assistant Superintendent, Buildings and Grounds
ROBERT B. MILLS, Manager, Department of Research Service

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ROBERT O. LEHY
MISS JOYCE B. LIMA
EDWARD F. MATTHEWS
BRUNO F. TRAPASSO
*HALLETT S. WAGSTAFF

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* Deceased.

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NORMAN MICHAUD, Head Chef
MRS. ELIZABETH KUIL, Supervisor, Dining Room
MRS. ELLEN T. NICKELSON, Supervisor, Dormitories
ALAN G. LUNN, Supervisor, Cottage Colony

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JACK TIBBS, University of St. Andrews, Scotland

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Raymon M. Glantz, New York University Medical School
Edward Gruberg, University of Illinois
Ronald R. Hoy, Stanford University
George J. Mpitsos, University of Virginia
Bert Shapiro, Harvard University
Denis A. Baylor, Yale University School of Medicine
Stephen R. Shaw, University of St. Andrews, Scotland

Rand Fellow, 1967

G. Adrian Horridge, University of St. Andrews, Scotland

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Antonellis, Blenda, Western Reserve University
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Boyle, Robert, Georgetown University Medical School
Brady, Arlene C., Mellon Institute
Brander, Keith M., Marine Science Laboratories, UCNW, Bangor
Brandhorst, Bruce P., University of California, San Diego
Bridges, Sandra H., Washington University
Bruner, Lynnel, The Johns Hopkins University
Bruzual, Iris B., Instituto Venezolano de Investigacions Cientificas, Venezuela
Burky, Albert, Syracuse University
Burns, Lawrence A., New York University
Campbell, Martin C., University of Chicago
Chasis, Sarah, New York University
Childress, Diana, University of Oregon
Collins, Sandra E., University of Kansas
Colville, Jean E., Tulane University
Cone, Margaret Virginia, University of Massachusetts
Connell, Penny May, Tulane University
Conta, Barbara A., University of Michigan
Copsey, Kathryn J., University of Miami
Cordell, Saundra, Columbia University

Dizon, Andrew, Yale University
Donahue, Helaine, Washington University School of Medicine
Doyle, Darrell J., University of Connecticut
Edds, Kenneth T., University of Rhode Island
Eigner, Elizabeth Ann, University of New Mexico Medical School
Epstein, Miles, Syracuse University
Ettienne, Earl M., State University of New York, at Albany
Fiel, Stanley, University of Connecticut
Fernandez, Hugo, Massachusetts Institute of Technology
Findley, Diane, University of Tennessee
Findley, Davis, University of Tennessee
Fitzjarrell, Austin T., Tulane University
Forman, David S., The Rockefeller University
Frick, Jane Allison, Columbia University
Gelfan, Janet I., Albert Einstein College of Medicine
Goldman, James E., University of Maryland
Goodenough, Daniel, Harvard Medical School
Goodyer, Paul R., Yale University
Greitzer, Naomi, Barnard College
Hall, Toni Jean, University of Michigan
Hamilton, John David, Massachusetts Institute of Technology
Hanson, Musetta, College of St. Mary of the Springs
Harris, Albert, Yale University
Harris, Edward M., Duke University
Harris, Elizabeth H., Yale University
Hart, Barbara Ann, Single Cell Research Foundation, Inc.
Hecht, Ralph, New York University
Hendrickson, Wayne, The Johns Hopkins University
Heymann, Peter W., Washington University
Hermolin, Joseph, University of Toronto, Canada
Hillman, Gilbert, Yale University
Hofman, Florence, City College of New York
Holt, Thomas, University of Connecticut
Householder, Sally, The Rockefeller University
Howell, Kathryn E., Rutgers, The State University
Huebner, Erwin, University of Massachusetts
Humphreys, Susie, University of California, San Diego
Irwin, M. Linda, Western Reserve University
Ivker, Frances S., Indiana University
Karten, Barbara, Columbia University
Katz, Eugene R., University of Cambridge, England
Katz, Jay A., Washington University School of Medicine
Kimball, Frances, Reed College
Kissin, Evelyn Anne, Columbia University
Kratowich, Nancy R., Columbia University
Krawchenko, John, State University of New York, Upstate Medical Center, Syracuse
Lang, Fred, University of Illinois
Lange, Mary T., University of Pennsylvania
Lankton, James, Harvard University
Lavorgra, Lorenzo, Washington University Medical School
Lawrence, Lyle, Marine Biological Laboratory
Lee, Thomas F., University of Connecticut
Leffert, Mark N., New York University School of Medicine
Lehman, William, Princeton University
Lemon, Stanley M., Princeton University
Letts, Pamela Jean, University of Toronto, Canada
Levy, Robert M., Washington University Medical School
Lipson, Robert Alan, VA Hospital, Brooklyn

Lipson, Stephen Jay, Yale University
Loverde, Vernon D., Washington University
Lucky, Paul A., Kenyon College
Mahanti, Mahendra, Columbia University
Marsh, Richard Charles, University of Maryland
Martinek, John J., Tulane University
Mason, John Montgomery, Jr., Marine Biological Laboratory
McGrath, Richard A., Northwestern University
McLaughlin, Jane A., The Institute for Muscle Research
Meltzer, Paul S., Dartmouth College
Merrill, Charlotte, Massachusetts Institute of Technology
Moffett, David F., Jr., Duke University
Moyer, Patricia Ellen, Radcliffe College
Mpitsos, George J., University of Virginia
Myhrman, Rolf V., Northwestern University
Nadol, Joseph B., Jr., The Johns Hopkins University School of Medicine
Natalini, John J., Northwestern University
Nichols, T. Richard, Brown University
O'Benar, John O., University of Illinois
Oberprilles, John, Tulane University
Oselka, Janet, Columbia University
Patterson, David, Brandeis University
Palmer, Jerry P., State University of New York, Upstate Medical Center, Syracuse
Parmentier, James Lawrence, University of California, Santa Barbara
Pederson, Thoru, Syracuse University
Percy, Jonathan, Columbia University
Perry, Maureen M., Trinity College, Washington, D. C.
Platt, Terry, Harvard University
Prior, Gwen, Brandeis University
Prusch, Robert D., Syracuse University
Pysar, Joanne, University of Connecticut
Quattropani, Steven, University of Massachusetts
Ransohoff, David F., Harvard College
Ravitz, Melvyn J., Columbia University
Rayner, Ellen P., Brandeis University
Rea, Ina Katherine, Washington University
Reynolds, George T., Princeton University
Ritch, Robert, Harvard University
Robbins, Marcia, Radcliffe College
Robertson, Lola E., American Museum of Natural History
Robinowitz, Bernard, Tulane University Medical School
Rosenblum, Judy Lynn, University of Vermont
Rossetto, Michalangelo A., The Rockefeller University
Ryder, Gary, State University of New York, Upstate Medical Center, Syracuse
Sage, Jean, Indiana University Medical School
Saltzman, Orah, Massachusetts Institute of Technology
Saul, David, The Johns Hopkins Medical School
Schoepf, Claude, Columbia University
Schwartz, Sue, Russell Sage College
Seldin, Edward B., Harvard School of Dental Medicine
Shapley, Robert, The Rockefeller University
Shaw, Stephen R., University of St. Andrews, Scotland
Siegel, Barry W., Rensselaer Polytechnic Institute
Sindelar, William F., Western Reserve University
Singer, Irwin, New York University
Sloane, Molla R., University of Virginia
Smarsh, Anne, VA Hospital, Boston
Squire, Richard D., North Carolina State University

Steinberg, William, University of Wisconsin
Stokes, Darrell R., University of Hawaii
Stoner, Larry C., Syracuse University
Strunk, Brian L., Princeton University
Suberman, Rick, University of North Carolina
Surgenor, Peter D., Hamilton College
Surver, William M., University of Notre Dame
Tam, Anthony, State University of New York, Syracuse
Tannenbaum, Bernice, National Institutes of Health
Tanner, Susane J., University of Hawaii
Tarr, Merrill, Duke University
Theriault, Jacques, University of Ottawa
Tuttle, Joan P., University of Rochester
Vary, James C., University of Wisconsin
Weians, Patricia, College of St. Mary of the Springs
Weiner, Beverly, Harvard Medical School
Wiksell, Audre, Washington University School of Medicine
Willenborg, David O., The Johns Hopkins School of Hygiene
Wilson, Frank J., University of Pittsburgh
Yoshioka, Paul M., Tulane University
Younger, W. B. Jerry, Washington University School of Medicine
Yutman, Marcia, Columbia University
Zuclich, Joseph A., Columbia University

Library Readers, 1967

Adelberg, Edward, Professor of Microbiology, Yale University
Allen, Garland E., Allston Burr Senior Tutor, Professor in the History of Science, Harvard University
Ball, Eric G., Professor of Biochemistry, Harvard Medical School
Battelle Memorial Institute, William F. Clapp Laboratories, Inc.
Benjamin, Thomas L., Assistant Member, American Medical Association
Bennett, Miriam F., Professor of Biology, Sweet Briar College
Berne, Robert M., Chairman and Professor, University of Virginia Medical School
Berry, Spencer J., Assistant Professor of Biology, Wesleyan University
Bersohn, Richard, Professor of Chemistry, Columbia University
Bodansky, Oscar, Vice President, Chief, Division of Biochemistry, Sloan-Kettering Institute for Cancer Research
Boettiger, Edward G., Professor of Physiology, University of Connecticut
Bridgman, Anna Josephine, Chairman and Professor of Biology, Agnes Scott College
Burbanck, W. D., Professor of Biology, Emory University
Butler, Elmer G., Osborn Professor of Biology, Princeton University
Carlson, Francis D., Professor of Biophysics, The Johns Hopkins University
Changeux, J. P., Maître-assistant Faculty Science, Institut Pasteur-Paris and Columbia University
Chase, Aurin M., Professor of Biology, Princeton University
Chauncey, Howard H., Chief, Research in Oral Diseases, Veterans Administration Central Office, Washington, D. C.
Clark, Arnold M., Professor of Biology, University of Delaware
Clement, A. C., Professor of Biology, Emory University
Clewe, Thomas H., Research Associate, Genetic & Developmental Disorders Division, Tulane University
Cohen, Edward P., Associate Professor of Microbiology & Medicine, Rutgers Medical School
Cooper, Jack Ross, Associate Professor of Pharmacology, Yale University School of Medicine
Crane, Robert K., Chairman and Professor of Physiology, Rutgers Medical School
Crowell, Sears, Professor of Zoology, Indiana University
de Villafranca, George W., Professor, Clark Science Center, Smith College
Edds, M. V., Jr., Professor of Biology, Brown University

Elliott, Alfred M., Professor of Zoology, University of Michigan
Fingerhut, Sr. Marion Robert, Graduate Student, Catholic University of America
Flavin, Rev. John W., Associate Professor of Biology, College of the Holy Cross
Flesch, Peter, Research Professor of Dermatology, University of Pennsylvania
Gabriel, Mordecai L., Chairman and Professor of Biology, Brooklyn College
Gibbs, Martin, Chairman and Professor of Biology, Brandeis University
Gitlin, David, Professor of Pediatrics, University of Pittsburgh School of Medicine
Glade, Richard W., Chairman and Professor of Zoology, University of Vermont
Gottschall, Gertrude Y., Assistant Professor of Medicine, The Mount Sinai Hospital
Greif, Roger L., Acting Chairman and Professor of Physiology, Cornell University Medical College
Gurewich, Vladimir, Assistant Professor of Clinical Medicine, New York College of Medicine
Handler, Philip, Chairman and Professor of Biochemistry, Duke University
Hurwitz, Charles, Chief, Basic Science Research Laboratory, VA Hospital, Albany
Ilan, Joseph, Assistant Professor of Biology, Temple University
Isselbacher, Kurt J., Professor of Medicine, Massachusetts General Hospital
Jacobs, Merkel H., Emeritus Professor of Physiology, University of Pennsylvania
Johnson, William H., Chairman and Professor of Biology, Rensselaer Polytechnic Institute
Kempton, Rudolf T., Professor of Biology, Vassar College
Kennedy, Eugene P., Hamilton Kuhn Professor of Biochemistry, Harvard Medical School
Keosian, John, Professor of Biology, Rutgers—The State University
Kiortsis, Vassili, Director and Professor of Zoological Laboratory & Museum, University of Athens, Greece
Kropf, Allen, Associate Professor of Chemistry, Amherst College
Levinthal, Cyrus, Professor of Biophysics, Massachusetts Institute of Technology
Lineaweaver, Thomas, III, Marine Biological Laboratory
Marks, Paul A., Associate Professor, Columbia University, College of Physicians & Surgeons
Mateyko, Gladys M., Professor of Biology, New York University
Moscona, A. A., Professor of Zoology, University of Chicago
Nachmansohn, David, Professor of Biochemistry, Columbia University, College of Physicians & Surgeons
Nason, Alvin, Professor of Biology, Associate Director of McCollum-Pratt Institute, The Johns Hopkins University
Novikoff, Alex B., Research Professor, Albert Einstein College of Medicine
Perlman, Gertrude E., Associate Professor, The Rockefeller University
Perlman, Hans Peter, Associate Professor of Immunology, Wenner-Gren Institute
Reichardt, Werner E., Director, Max-Planck-Institut für Biologie, Germany
Rowland, Lewis P., Professor of Neurology, Columbia University, College of Physicians & Surgeons
Rugh, Roberts, Associate Professor of Radiology, Columbia Medical Center
Sager, Ruth, Professor, Hunter College
Schlesinger, R. Walter, Chairman of Department of Microbiology and Assistant Dean, Rutgers Medical School
Schopf, Thomas J. M., Systematics-Ecology Program, Marine Biological Laboratory
Silman, Israel, Research Associate, Columbia University, College of Physicians & Surgeons
Simon, Joseph L., Systematics-Ecology Program, Marine Biological Laboratory
Spector, Abraham, Assistant Professor of Ophthalmology, Columbia University
Sperelakis, Nick, Professor of Physiology, University of Virginia School of Medicine
Stetten, DeWitt, Dean, Rutgers Medical School
Stetten, Marjorie R., Research Professor of Experimental Medicine, Rutgers Medical School
Summers, William C., Systematics-Ecology Program, Marine Biological Laboratory
Travis, Dorothy F., Assistant Biologist, Massachusetts General Hospital
Twarog, Betty M., Assistant Professor of Biology, Tufts University
Wade, Barry A., Systematics-Ecology Program, Marine Biological Laboratory
Wainio, Walter, Professor of Biochemistry, Rutgers-The State University
Wallace, Stanley L., Associate Director of Medical Service and Associate Clinical Professor of Medicine, Jewish Hospital and State University of New York, Downstate Medical Center

Wheeler, George E., Associate Professor of Biology, Brooklyn College
Whiting, Anna R., Consultant, Oak Ridge National Laboratory
Wichterman, Ralph, Professor of Biology, Temple University
Wilson, Thomas Hastings, Associate Professor of Physiology, Harvard Medical School
Yntema, Chester L., Professor of Anatomy, State University of New York, at Syracuse
Young, David K., Systematics-Ecology Program, Marine Biological Laboratory
Zacks, Sumner I., Associate Professor of Pathology, University of Pennsylvania School of Medicine & Pennsylvania Hospital
Zullo, Victor A., Systematics-Ecology Program, Marine Biological Laboratory

Students, 1967

All students listed completed the formal course program, June 12–July 22. Asterisk indicates students completing post-course research program, July 23–September 2.

ECOLOGY

Anderson, Robert, Syracuse University
*Beard, Elizabeth, University of Wisconsin
Brodsky, Diane, Northwestern University
Fox, John, The Johns Hopkins University
*Hall, John, University of North Carolina
*Halverson, Roger, Syracuse University
Heithaus, Earl, Kenyon College
*Keeley, Rosemary, Yale University
Kircher, Ann, Duke University
McCormack, Brian, University of Miami
*Meade, James, Cornell University
*Miller, Nancy, The Johns Hopkins University
*Nyblade, Carl, Oberlin College
Schneider, Douglas, Amherst College
*Sherwood, Marjorie, University of California, Los Angeles
Shulman, Richard, University of Illinois
*Spoon, Donald, Emory University
White, Alan, Harvard University
Wurster, Charles, State University of New York

EMBRYOLOGY

Anderson, Paul N., National Institutes of Health, National Cancer Institute
*Astrin, Kenneth H., University of California, San Diego
Boettiger, David, Western Reserve University
Browder, Leon W., University of Minnesota
*Fry, Barry J., Massachusetts Institute of Technology
Garfield, Sanford, University of Illinois
*Granholm, Norman A., University of Oregon
Hake, Jonathan C., Washington University
Helfenstein, Myrna C., Washington University
Householder, Sally C., The Rockefeller University
*Huz, Shulamith, New York University, Washington Square College
*Kedes, Laurence H., Massachusetts Institute of Technology
*Kerby, Catherine J., Institute of Molecular Evolution, University of Miami
*McMorris, F. Arthur, Yale University
*Phillips, Dwight E., Tulane University
Rechsteiner, Martin C., The Johns Hopkins University
Rozin, Paul, University of Pennsylvania
Watson, Lesley F., Yale University

*Webster, Dale A., Harvard Medical School & Massachusetts General Hospital
Wesley, Ronald D., Oregon State University

MARINE BOTANY

Anderson, Daryl J., Washington University
*Archibald, Patricia Ann, University of Texas
*Campbell, Louise, University of Colorado
Durkin, Sister Eileen M., RSM, University of California, Berkeley
*Erlbaum, Patricia A., Fordham University
Giesmann, Larry Allen, University of Kentucky
*Hendrix, Mary L., Wellesley College
Kazama, Frederick, University of California, Berkeley
Lee, Robert Edward, University of Massachusetts
Lee, Robert Wingate, State University of New York, Stony Brook
McCracken, Michael D., Indiana University
*Menden, Trina M., Goucher College
*Mullins, John Q., University of Tennessee
*Redemske, Joyce, University of Illinois
*Roy, Victoria M., College of New Rochelle
Spoon, Jeanette A. H., Emory University
Stewart, Dorothy J., Drew University
Tucker, Lynne, Oberlin College
Vande Berg, Warren James, Indiana University
*Villalard, Martine, University of Rhode Island

PHYSIOLOGY

*Abzug, Charles, New York Medical College
*Anderson, Carl W., Washington University
Asbell, Mary Ann, University of Georgia
*Bellemare, Guy, University of Montreal
*Blumenthal, Thomas, The Johns Hopkins University
Chan, Teh-Sheng, Yale University
*Chen, Victor Kai-Hwa, State University of New York, at Buffalo
Dearman, Henry H., University of North Carolina
*Eberhard, Carolyn, Boston University
Elliott, Donald A., The Rockefeller University
Feher, George, University of California, San Diego
Gleich, Gerald J., Mayo Clinic
*Hillman, Peter, Weizmann Institute, Rehovoth, Israel
*Horch, Kenneth W., Yale University
*Howell, William H., Columbia University
*Kirschner, Marc, The Rockefeller University
Klock, Peter A., The Johns Hopkins University
*Kowit, Joel D., Brandeis University
Munse, Patricia A., Harvard University
*Nealson, Kenneth H., University of Chicago
*Nickerson, Kenneth W., University of Cincinnati
Nossal, Ralph J., National Institutes of Health
Osborn, Mary, Pennsylvania State University
Pardue, Mary Lou, Yale University
*Piper, Judith A., Marquette University
Reif, Frederick, University of California, Berkeley
*Sekaer, Christina M., Western Reserve University
Shih, Agnes An-Ya, Yale University
*Swenarchuk, Lauren C., Western Reserve University
*Taber, Robert L., Jr., University of Pittsburgh

Auditors: Donald Graham, Case Western Reserve University
Jerome S. Harris, Duke University
Martin Posner, Yale University

INVERTEBRATE ZOOLOGY

Asher, Kenneth D., Ohio Wesleyan University
Bailey, Barbara Ann, University of Oklahoma
Bailey, Kaniaulono Helen, Acadia University
Balderston, W. Lloyd, Long Island University
Bennett, Albert F., University of Michigan
Billings, Susan M., Harvard Medical School
Bruns, Romaine R., Harvard Medical School
Cheney, Clarissa M., Goucher College
*Collier, Marjorie M., Brooklyn College
*Cramer, Nancy Marie, George Washington University
Cumbie, Peter M., Yale University
Ebner, Ford Francis, Brown University
Engelhardt, Charles D., Drew University
Erickson, Gregory F., University of Illinois
Ettienne, Earl Manual, State University of New York, at Albany
Fingerhut, Sister Marion Robert, Catholic University of America
Gabe, Philip R., University of California, Berkeley
Henninger, Ann Louise, Wilson College
*Hunter, R. Douglas, Syracuse University
*Johnston, Michael A., Colorado College
*Krasilovsky, George H., University of Oregon
Kucinski, Helen Mary, University of Massachusetts
Kuprys, Jeannie Genovaite, Connecticut College
Lucky, Paul Andrew, Kenyon College
*McMahon, Robert F., III, Syracuse University
Myers, Allen Cowles, University of Rhode Island
Nace, A. George, III, Cornell University
Natalini, John Joseph, Northwestern University
Newton, William Donald, University of North Carolina
Oberdorfer, Michael D., University of Wisconsin
*Paracer, S. M., University of Massachusetts
Pillow, Christina Louise, Biological Abstracts
Rhys, Ann Ferrier, Oberlin College
*Rice, Harbert Vivian, Jr., Harvard University
Ritzi, Earl Michael, University of Massachusetts
*Smith, Elnora Harriet, University of Michigan
Tirey, Sandra Lee, Rice University
*Waddell, James L. F., Glasgow University, Scotland
Williams, Gabriel O., University of California, Berkeley
Wright, Virginia K., Emory University
*Young, Janice E., Goucher College

4. Fellowships and Scholarships, 1967

The Lucretia Crocker Scholarship:
Roger C. Halverson, Marine Ecology Course
Martine Villalard, Marine Botany Course

The James W. Mavor Scholarship:
James L. F. Waddell, Invertebrate Zoology Course

The Turtox-Croasdale Scholarship:
PATRICIA ERLBAUM, Marine Botany Course
Gary N. Calkins Memorial Scholarship:
HARBERT VIVIAN RICE, JR.
ELNORA HARRIET SMITH
James Watt Mavor Scholarship:
JAMES L. F. WADDELL

5. TRAINING PROGRAM

FERTILIZATION AND GAMETE PHYSIOLOGY RESEARCH TRAINING PROGRAM

I. INSTRUCTORS

CHARLES B. METZ, University of Miami, Program Chairman
C. R. AUSTIN, Tulane University, Delta Regional Primate Center
DAVID BISHOP, Carnegie Institution of Washington
GEORGE G. BROWN, University of Miami
GIOVANNI GIUDICE, University of Palermo, Italy
GERTRUDE HINSCH, University of Miami
ALBERTO MONROY, University of Palermo, Italy

II. TRAINEES

CLAUDIO BARROS, University of Tulane, Delta Regional Primate Center
GRACIELA CANDELAS, University of Puerto Rico
JOHN CHAMBERLAIN, Princeton University
CHARLES FOURTNER, Carroll College
VITAUTS KALNINS, NATO Science Post-Doctoral, Harvard University
LAWRENCE KOEHLER, Central Michigan University
NORMAN LAZAROFF, Yale University
PATRICIA OLDS, Washington University
RICHARD PELTZ, University of Pennsylvania
HARRY ROY, The Johns Hopkins University
LEONARD SCHECHTMAN, The Johns Hopkins University
W. WINSLOW SCHRANK, University of Maryland
ROSS SHOGER, Associate Professor, Carlton College
PHILIP SKEHAN, Yale University
RICHARD WEISENBERG, University of Chicago
NATILLE HEADRICK, University of Tennessee

III. LECTURES

DAVID BISHOP	Testicular sorbitol dehydrogenase in relation to differentiation and modification of germinal epithelium
I. FRIEDMAN	Cytology of fertilization in algae
A. CLARK	Aberrant fertilization types in the wasp *Habrobracon*
L. BARTH	Parallelism between induction and egg activation
P. SOUPART	Studies on the hormonal control of rabbit sperm capacitation
D. PATANELLI	Physiological control of spermatogenesis
C. VILLEE	The role of RNA in mediating the effects of testosterone
H. GRUNDFEST	The egg as an electrophysiological system
J. MARSH	Egg cell lipids—the lighter side of reproduction?
L. PIKO	Some studies of membranes during fertilization
A. MACLAREN	Fertilization in the mouse
M. BEDFORD	Some electron microscopic studies of fertilization

6. Tabular View of Attendance, 1963–1967

	1963	*1964*	*1965*	*1966*	*1967*
Investigators—Total	490	512	572	555	590
Independent	261	273	284	287	313
Library Readers	51	47	62	77	78
Research Assistants	178	192	227	191	199
Students—Total	124	126	128	126	132
Invertebrate Zoology	40	40	41	37	41
Embryology	20	20	20	22	20
Physiology	28	30	30	29	31
Botany	20	19	20	18	20
Ecology	16	17	17	20	20
Trainees—Total		30	34	29	16
Nerve-Muscle		7	7	7	
Comparative Physiology		7	11	6	
Fertilization & Gamete		16	16	16	16
Total Attendance	614	668	734	710	738
Less persons represented in two categories	5	7	4	0	4
	609	661	730	710	734
Institutions Represented—Total	120	140	218	198	177
By Investigators	83	117	142	105	114
By Students	73	23	76	76	74
By Library Readers				47	74
By Research Assistants				80	75
Foreign Institutions Represented	21	32	27	28	29
By Investigators	15	28	25	20	24
By Students	6	4	2	4	3
By Library Readers				1	3
By Research Assistants				4	7

7. Institutions Represented, 1967

Agnes Scott College
Albert Einstein College of Medicine
American Medical Association, Chicago
American Museum of Natural History
Amherst College
Argonne National Laboratory
Augustana College
Barnard College
Battelle Memorial Institute
Biological Information Service, Philadelphia
Boston University
Brandeis University
Brooklyn College, City University of New York
Brown University
Bryn Mawr College
California Institute of Technology
California, University of, at Berkeley
California, University of, at Los Angeles
California, University of, at Riverside
California, University of, at San Diego
California, University of, at Santa Barbara
Carlton College
Carnegie Institution of Washington
Catholic University of America
Central Michigan University
Chicago, University of
Cincinnati, University of
Clapp, Wm. F., Laboratories
Colgate University
Colorado College
Colorado, University of, Medical School
Columbia Medical Center
Columbia University
Columbia University, College of Physicians & Surgeons
Connecticut College
Connecticut, University of
Cornell University
Cornell University Medical College

Dartmouth College
Delaware, University of
Delta Regional Primate Research Center, Tulane University
Drew University
Duke University
Emory University
Fordham University
Georgetown University
Georgetown University Medical School
George Washington University
Georgia, University of
Goucher College
Hamilton College
Harvard College
Harvard Medical School
Harvard School of Dental Medicine
Harvard University
Hawaii, University of
Holy Cross, College of the
Hunter College, City University of New York
Illinois Institute of Technology
Illinois, University of
Indiana University
Indiana University Medical Center
Institute for Cancer Research, Philadelphia
Institute for Muscle Research, Woods Hole
Jewish Hospital, Brooklyn
Johns Hopkins University, The
Johns Hopkins University School of Hygiene & Public Health, The
Johns Hopkins University School of Medicine, The
Kansas, University of
Kentucky, University of
Long Island University
Marquette University
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts, University of
Mayo Clinic
McLean Hospital Research Laboratory
Mellon Institute, The
Miami University at Ohio
Miami, University of
Miami, University of, Institute of Molecular Evolution
Michigan State University
Michigan, University of
Minnesota, University of
Mount Holyoke College
Mount Sinai Hospital
National Institutes of Health
New Mexico, University of, Medical School
New Rochelle, College of
New York, City College of
New York Medical College
New York, State University of, Albany
New York, State University of, Buffalo
New York, State University of, Stony Brook
New York, State University of, College of Medicine, Upstate Medical Center, Syracuse
New York University
New York University, College of Dentistry
New York University, Institute of Environmental Medicine
New York University Medical School
New York University, Washington Square College
North Carolina State University at Raleigh
North Carolina, University of
Northeastern Illinois State College
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oberlin College
Ohio Wesleyan University
Oklahoma, University of
Oregon State University
Oregon, University of
Pennsylvania Hospital
Pennsylvania State University
Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pittsburgh, University of
Pittsburgh, University of, School of Medicine
Princeton University
Providence College
Queens College, City University of New York
Radcliffe College
Reed College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rice University
Rochester, University of
Rochester, University of, School of Medicine & Dentistry
Rockefeller University, The
Russell Sage College
Rutgers, The State University, New Brunswick
Rutgers University, Newark
Rutgers University Medical School
St. John's College
St. Louis University
St. Mary of the Springs, College of
San Diego State College
Simmons College
Single Cell Research Foundation, Inc.
Sloan-Kettering Institute for Cancer Research
Smith College
Smithsonian Institution
Southern Connecticut State College

Stanford University
Sweet Briar College
Syracuse University
Temple University
Tennessee, University of
Texas, University of
Toledo, University of
Trinity College, Hartford
Trinity College, Washington, D. C.
Tufts University
Tulane University
Vassar College
Vermont, University of
Veterans Administration Center, Los Angeles
Veterans Administration Central Office, Washington, D. C.
Veterans Administration Hospital, Albany
Veterans Administration Hospital, Boston
Veterans Administration Hospital, Brooklyn
Veterans Administration Hospital, Pittsburgh
Virginia Polytechnic Institute
Virginia, University of
Virginia, University of, Medical School
Wards Natural Science Company
Washington University, St. Louis
Washington University School of Medicine
Wellesley College
Wesleyan University
Western Reserve University
Western Reserve University School of Medicine
Wilson College
Wisconsin, University of
Yale University
Yale University School of Medicine
Yeshiva University

Foreign Institutions Represented, 1967

Acadia University, Nova Scotia
Athens, University of, Greece
Bedford College, University of London
Biologische Anstalt Helgoland, West Germany
British Columbia University, Vancouver
Cambridge, University of, England
Chile, University of
Dalhousie University, Nova Scotia
Ehime University, Japan
Geneva, University of, Switzerland
Genoa University, Italy
Glasgow University, Scotland
Institut Pasteur, Paris
Instituto Venezolano de Investigaciones Cientificas, Venezuela
International Laboratory of Genetics and Biophysics, Italy
Ljubljana University, Yugoslavia
Marine Science Laboratory, England
Max-Planck Institut für Biologie, Germany
Medical Research Council, England
MRC Laboratory of Molecular Biology, England
Montreal University, Canada
Okayama University, Japan
Ottawa, University of, Canada
Palermo, University of, Italy
Puerto Rico, University of
St. Andrews, University of, Scotland
Teheran University, Iran
Tohoku University, Japan
Toronto, University of, Canada
University College, London, England
Weizmann Institute, Israel
Wenner-Gren Institute, Sweden

Supporting Institutions, Agencies, and Individuals

Associates of the Marine Biological Laboratory
Atomic Energy Commission
Luther J. Carter
Mr. & Mrs. W. Van Alan Clark
Dr. William D. Curtis
Mrs. Alexander Forbes
The Ford Foundation
Mr. John A. Gifford
Mr. W. T. Golden
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8. Friday Evening Lectures, 1967

July 7
Alberto Monroy .... Recent Studies on the Molecular Aspects of the Activation of the Egg
University of Palermo, Italy

July 13	Werner Reichardt Max-Planck-Institut für Biologie Alexander Forbes Lecturer at the Marine Biological Laboratory	On Structure and Function of Retina and Lamina in Diptera
July 14	Werner Reichardt	Analysis of Optomotor Responses
July 21	Robert A. Good University of Minnesota Medical School	Development and Involution of Immunologic Capacity
July 28	Philip Abelson Editor, Science	Chemical Events on the Primitive Earth
August 4	Michail Fischberg Université De Geneva, Switzerland Senior Lalor Fellow	Information and Development in *Xenopus laevis*
August 11	G. Adrian Horridge University of St. Andrews, Scotland Rand Fellow	The Action of the Retina in Arthropod Compound Eyes of the Apposition Type
August 18	Kenneth S. Cole National Institutes of Health (NINDB)	Membrane Watching
August 25	Peter Perlmann University of Stockholm, Sweden F. R. Lillie Fellow	Aggressor Function of the Vertebrate Lymphocytes: Its Mechanism and Biological Significance

9. Tuesday Evening Seminars, 1967

July 11	K. Ranga Rao Milton Fingerman C. K. Bartell	Hormones Controlling the White Chromatophores of the Fiddler Crab, *Uca pugilator* Is There a Physiological Limit to the Response of Melanophores in *Uca* to the Melanin-dispersing Hormone?
	R. B. Loftfield E. A. Eigner	Comparative Biochemistry of RNA and tRNA Ligase Reactions
July 18	Norman B. Rushforth	Behavioral Responses of Isolated Tentacles of Hydra
	Joseph L. Simon	Behavioral Aspects of *Histriobdella homari*, an Annelid Commensal of the American Lobster

Daniel S. Grosch	Biosatellite Experiments I. General Design of Experiments II. *Habrobracon* Investigations

July 25

S. Ehrenpreis	Modification of Smooth Muscle Receptors by Heat and Urea
S. Lerman S. Zigman	The Relationship Between Soluble and Insoluble Protein in the Lens
P. Rosenberg	Demonstration of Phospholipid Splitting as the Factor Responsible for Increased Permeability and Block of Axonal Conduction Induced by Snake Venom—Study on Squid Axons
F. C. G. Hoskin	Studies on the Penetration of Some Organophosphorus Cholinesterase Inhibitors into the Squid Giant Axon

August 1

Richard Cone	Early Receptor Potential: Photoreversible Charge Displacement in Rhodopsin
Bruce Goldstein	Source of the Early Receptor Potential in the Isolated Frog Retina
Joel E. Brown and Tom Smith	Photoelectric Potentials in the *Limulus* Eye
W. A. Hagins and R. E. McGaughy	The Origin of Fast Photoelectric Effects in the Squid Retina

August 8

W. D. Hummon	Interstitial Marine Gastrotrichs from Woods Hole, Massachusetts
B. A. Wade	Ecological Studies of New England Nudibranchs
P. Person and H. Zipper	Water in Biological Systems I. Solubilization of Yeast and Heart Mitochondrial Cytochrome Oxidase and Cytochrome *b* by Synthetic Zeolites
J. Felton H. Zipper and P. Person	Sequential Solubilization of Mitochondrial Cytochrome System Components at Alkaline pH's

August 15

W. W. Schrank R. L. Shoger L. M. Schachtman D. W. Bishop	Electrically Induced Spawning in the Male and Female Horseshoe Crab, *Limulus polyphemus*
M. Lochhead and J. H. Lochhead	The Development of Oocytes in the Brine Shrimp, *Artemia*
L. C. Stoner	Temperature Acclimation in *Tetrahymena pyriformis*
R. H. Green K. D. Hobson S. L. Santos	Patterns of Invertebrate Distribution in the Barnstable Harbor Intertidal Area
J. A. Miller, Jr.	Temperature, pH and Glucose in Experimental Asphyxia of the Newborn

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Including Action of 1967 Annual Meeting

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Wilson, Dr. May G.
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Wrinch, Dr. Dorothy
Yntema, Mrs. Chester L.
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V. REPORT OF THE LIBRARIAN

Photocopying has become a major activity of the Library. During the summer months two Xerox machines were running constantly and an extra employee was hired to operate them. The machines are located in a library room on the third floor of the Lillie building.

The copying service has also doubled the interlibrary loan requests processed over the previous year. During 1967 we received 4,417 separate requests for articles contained here in the library. The requests were sent from universities, government agencies, hospitals, industrial firms and individual students and researchers. We made 342 requests from other libraries for the use of investigators here. Our list of "Serial Publications," containing approximately 4,000 separate titles, has been placed in over 800 libraries throughout the country.

Over 2,000 volumes were sent to the bindery in 1967 and the total holdings are now 133,447. This figure does not include the reprint floor.

Total number of serial titles in library		3,962
Number received currently		2,292
On subscription	921	
On exchange	945	
On gift basis	345	
Number of reference books added in 1966		532
Received from book exhibitors	167	
Total number of reprints in collection		240,417
Number added in 1967		2,197

Respectfully submitted,
Jane Fessenden,
Librarian

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 21, 1967, amounted to $2,313,618, and the corresponding securities are entered in the books at a value of $1,397,833. This compares with values of $2,249,448 and $1,326,668, respectively, at the end of the preceding year. The average yield on the securities was 3.99% of the market value and 6.6% of the book value. Uninvested principal cash was $4,728. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1967, amounted to $710,152 as compared to book values of $618,416. These figures compare with values of $701,945 and $664,523, respectively, at the close of the preceding year. The average yield on the securities was 3.93% of the market value and 4.5% of the book value. Uninvested principal cash was in the amount of $387.

In October, the Laboratory sold its holdings in General Biological Supply House for $2,664,212, realizing a profit of $2,651,512. The proceeds of this sale are currently invested in short-term commercial notes.

The proportionate interest in the Pool Fund Account of the various Funds, as of December 31, 1967, is as follows:

Pension Funds	19.992%
General Laboratory Investment	24.638%
F. R. Lillie Memorial Fund	2.685%
Anonymous Gift	.921%
Other:	
Bio Club Scholarship Fund	.695%
Rev. Arsenius Boyer Scholarship Fund	.851%
Gary N. Calkins Fund	.798%
Allen R. Memhard Fund	.156%
Lucretia Crocker Fund	2.907%
E. G. Conklin Fund	.491%
Jewett Memorial Fund	.258%
M. H. Jacobs Scholarship Fund	.351%
Herbert W. Rand Fellowship	24.501%
Mellon Foundation	11.727%
Mary Rogick Fund	2.571%
Swope Foundation	6.458%

Donations from MBL Associates for 1967 amounted to $8,010 as compared with $7,595 for 1966. A gift of a boat was received from Charles L. Morse, Jr., valued at $12,000. Unrestricted gifts from foundations, societies and companies amounted to $4,408.

Donations received during the year, donated specifically to the new laboratory under construction, amounted to $5,900.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
11 NIH	3 NIH	3 NIH
4 NSF	2 NSF	3 NSF
2 FORD		1 FORD
1 Whitehall		2 ONR
2 ONR		1 AEL
—	—	—
20	5	10

The rate of overhead on continuing grants from Federal agencies is effectively 20% of the amounts expended. For new Federally funded research grants a provisional rate of 25% has been negotiated. However, the Laboratory is required to make an identifiable contribution to the total cost of each research project. The overhead on those grants wherein provision therefore was made in the funds awarded within the year, amounted to $95,874 as compared with $85,822 for the preceding year. A proposal to NSF for determining an indirect cost rate for the current year is in course of preparation.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1967, the related statement of operating expenditures and income and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory of the year ended December 31, 1966.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1967 and 1966 and the results of its operations for the years then ended on a consistent basis.

The supplementary schedules included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.

Boston, Massachusetts
March 29, 1968 Lybrand, Ross Bros. and Montgomery

It will be noted from the operating statement that the Laboratory activities for the year under review, amounted to a figure of a little over 1.6 million dollars, which amount is comparable to last years results.

Alexander T. Daignault,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1967 and 1966

Investments

	1967	1966
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1967—$2,313,618)	$1,397,833	$1,326,668
Cash	4,728	1,105
	1,402,561	1,327,773
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1967—$710,152) less $5,728 temporary investment of current fund cash	612,688	658,795
Other investments	2,803,850	121,370
Cash	387	50,108
Accounts receivable	54	1,393
Due from current fund	87,542	
	$4,907,082	$2,159,439

Plant Assets

	1967	1966
Land, buildings, library and equipment (note)	5,695,732	5,649,105
Less allowance for depreciation (note)	1,624,698	1,537,471
	4,071,034	4,111,634
Construction in progress	110,042	28,453
Cash		6,056
Short-term investments, at cost	50,000	50,000
Due from current funds	15,365	
	$4,246,441	$4,196,143

Current Assets

	1967	1966
Cash	107,483	48,860
Temporary investment in pooled securities	5,728	5,728
U. S. Treasury bills, at cost		58,219
Accounts receivable (U. S. Government, 1967—$83,619; 1966—$55,247)	139,771	127,817
Inventories of supplies and bulletins	46,372	40,525
Other assets	8,698	31,580
Due to plant funds	(15,365)	
Due to endowment funds	(87,542)	
	$ 205,145	$ 312,729

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1967 and 1966

Invested Funds

	1967	*1966*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,402,561	$1,327,773
Endowment funds for awards and scholarships:		
Principal	427,663	427,663
Unexpended income	32,738	24,297
	460,401	451,960
Unrestricted funds functioning as endowment	2,857,890	206,378
Retirement fund	169,833	150,703
Pooled investments—accumulated gain	16,397	22,625
	$4,907,082	$2,159,439

Plant Funds

Funds expended for plant, less retirements	5,805,774	5,677,558
Less allowance for depreciation charged thereto	1,624,698	1,537,471
	4,181,076	4,140,087
Unexpended plant funds	65,365	56,056
	$4,246,441	$4,196,143

Current Liabilities and Funds

Accounts payable and accrued expenses	1,947	69,385
Advance subscriptions	15,210	16,746
Unexpended grants—research	32,353	61,780
Unexpended balances of gifts for designated purposes	23,899	22,281
Current fund	131,736	142,537
	$ 205,145	$ 312,729

Note—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1967 and 1966

Operating Expenditures

	1967	*1966*
Research and accessory services	$ 323,854	$ 329,066
Instruction	219,345	179,964
Library and publications (including book purchases—1967, $34,626; 1966, $34,082)	96,850	105,857
Direct costs on research grants	451,756	466,824
Direct costs on institution support grants	89,228	110,993
	1,181,033	1,192,704
Administration and general	182,008	139,888
Plant operation and maintenance	145,900	147,271
Dormitories and dining	203,058	198,668
Additions to plant from current fund		34,528
	1,711,999	1,713,059
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	87,226	88,786
	1,624,773	1,624,273

Income

	1967	*1966*
Research fees	99,716	97,186
Accessory services (including sales of biological specimens—1967, $42,247; 1966, $34,551)	123,499	133,416
Instruction fees	27,350	28,750
Library fees, bulletins, subscriptions and other	74,131	64,705
Dormitories and dining income	140,257	141,393
Grants for support of institutional activities:		
Instruction and training	220,451	177,825
Support services	89,228	110,993
General	132,097	130,750
Reimbursements and allowances for direct and indirect costs on specific research grants	526,570	534,909
Gifts used for current expenses	24,418	17,125
Investment income used for current expenses	180,955	162,262
	1,638,672	1,599,314
Excess current income (expenditures)	$ 13,899	($ 24,959)

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1967

	Balance December 31, 1966	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance December 31, 1967*
Invested funds	$2,159,439	$2,746,799	$200,383	$ 179,079	$ 20,460	$4,907,082
Unexpended plant funds	$ 56,056	87,516	3,382		81,589	$ 65,365
Unexpended research grants	$ 61,780	938,919		968,346		$ 32,353
Unexpended gifts for designated purposes	$ 22,281	28,825		24,418	2,789	$ 23,899
Current fund	$ 142,537	13,899(1) (12,000)(2) (12,700)(3)				$ 131,736
		$3,791,258	$203,765	$1,171,843	$104,838	

Gifts	34,753
Grant for facilities construction	81,589
Grant for research, training and support	938,919
Appropriated from current income and other	26,726
Net gain on sale of securities	2,720,072
(1) Excess of current income over expenditures	13,899
(2) Gift of boat transferred to plant funds expended	(12,000)
(3) Loss on sale of gift in kind	(12,700)
	$3,791,258

Expended for new Laboratory planning	81,589
Scholarship awards	6,530
Payments to pensioners	13,360
Other	3,359
	$104,838

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1967

	Cost	*% of Total*	*Market Quotations*	*% of Total*	*Investment Income 1967*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	2.2	$ 24,430	1.3	$ 1,250
Corporate bonds	674,563	59.5	555,267	29.2	28,361
Preferred stocks	102,916	9.1	107,375	5.6	2,595
Common stocks	331,875	29.2	1,214,818	63.9	45,455
	1,134,419	100.0	1,901,890	100.0	77,661
General educational board endowment fund:					
U. S. Government securities	53,112	20.2	51,791	12.6	3,202
Other bonds	129,971	49.3	103,843	25.2	4,264
Preferred stocks	31,914	12.1	37,275	9.1	370
Common stocks	48,417	18.4	218,819	53.1	6,826
	263,414	100.0	411,728	100.0	14,662
Total securities held by Trustee	$1,397,833		$2,313,618		$ 92,323
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	2,000	.3	1,645	.2	1,229
Corporate bonds	227,194	36.8	181,380	25.6	9,838
Common stocks	389,222	62.9	527,127	74.2	16,845
	618,416	100.0	$ 710,152	100.0	27,912
Less temporary investment of current fund cash	(5,728)				(266)
	612,688				27,646
Other investments:					
U. S. Government securities	27,938				1,133
Other bonds	15,029				750
Preferred stocks	3,448				135
Common stocks	46,186				57,174
Real estate	17,549				
Short-term commercial notes	2,693,700				30,392
	2,803,850				89,584
Total investments of other endowment and unrestricted funds	$3,416,538				117,230

Total		209,553
Custodian's fees charged thereto		(9,170)
Investment income distributed to invested funds		200,383
Plant investments:		
Federal agency and corporate bonds	$ 50,000	3,382
Current investments:		
U. S. Treasury bills		1,610
Temporary investment in pooled securities	$ 5,725	266
		1,876
Total investment income		$205,641

STUDIES ON MEMBRANE TRANSPORT. II. THE ABSORPTION OF ACETATE AND BUTYRATE BY HYMENOLEPIS DIMINUTA (CESTODA)[1]

C. ARME AND C. P. READ

Department of Biology, Rice University, Houston, Texas 77001

Lipid metabolism in tapeworms has been studied recently by several investigators (Fairbairn *et al.,* 1961; Harrington, 1965; Ginger and Fairbairn, 1966a, b; Jacobsen and Fairbairn, 1967; Lumsden and Harrington, 1966; McMahon, 1961), and there is abundant evidence that *Hymenolepis diminuta* absorbs fatty acids from the external medium and incorporates them into lipid components of the tissues. Von Brand's hypothesis (1966), that the fatty acids in tapeworm tissues are waste products of carbohydrate metabolism, has not been supported by careful *in vivo* and *in vitro* experiments (Ginger and Fairbairn, 1966b; Jacobsen and Fairbairn, 1967), and all direct available evidence suggests that the lipids of *H. diminuta* are synthesized from fatty acids absorbed from the environment. On the other hand, although there is considerable evidence for the mediated absorption of amino acids, sugars, purines, and pyrimidines by this worm (reviewed by Read, 1966), there seem to be no published studies dealing with mechanisms for absorption of fatty acids by *H. diminuta* or other tapeworms. The present investigation was undertaken to characterize systems by which these presumably important nutrients may enter the tissues of some parasitic organisms.

MATERIALS AND METHODS

Read, Rothman and Simmons (1963) described techniques for the maintenance and experimental manipulation of the tapeworm, *Hymenolepis diminuta.* With few exceptions these methods were adopted during the present investigation.

Young male rats (Holtzman Rat Co., Madison, Wisconsin), weighing 80–100 g. at the time of infection, were used as hosts in all experiments; animals received a diet of Purina Laboratory Chow. Ten days following infection with 30 cysticercoids (obtained from previously infected *Tenebrio molitor*), the rats were killed and the intestines removed. Parasites were flushed from the intestine with Krebs-Ringer solution containing 25 m*M* tris(hydroxymethyl)aminomethane-maleic acid buffer at pH 7.4. This solution (KRT) was used for all subsequent washes and, except as otherwise stated, as a solute in experimental incubations. After removal from the intestine, worms were washed and randomized in groups of five. They were then preincubated in 10 ml. KRT for a period of 30 minutes in a shaker bath at 37° C., after which each five-worm sample was transferred to 4 ml. of incubation solution for a period of one minute. Following incubation worms were washed in KRT, blotted on hard filter paper and dropped into 5 ml. 70% ethanol. After

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[1] This work was supported by grants from the National Institutes of Health, U. S. Public Health Service, AI-01384 and 5 TI AI 106.

18–24 hrs. the worms were removed from the ethanol, dried overnight in an oven at 90° C. and weighed. Aliquots of the ethanol extract were mixed with 0.5 ml. of a 5 m*M* sodium bicarbonate solution prior to drying on aluminum planchettes and counting on a gas flow counter. Uptake data are based on the amount of radioactive material extracted in 70% ethanol and are expressed as μmoles/g./min., using the ethanol-extracted dry weight.

Sodium acetate and sodium butyrate labelled with ^{14}C at the C-1 position were obtained from New England Nuclear Co. and Nuclear Chicago Co., respectively. Unlabelled sodium salts of valeric acid (Eastman Organic Chemicals) and octanoic acid (Matheson, Coleman, and Bell) were prepared by titration of the appropriate acid to pH 7.6 with sodium hydroxide. Formate, acetate, propionate and butyrate were purchased as sodium salts of reagent grade.

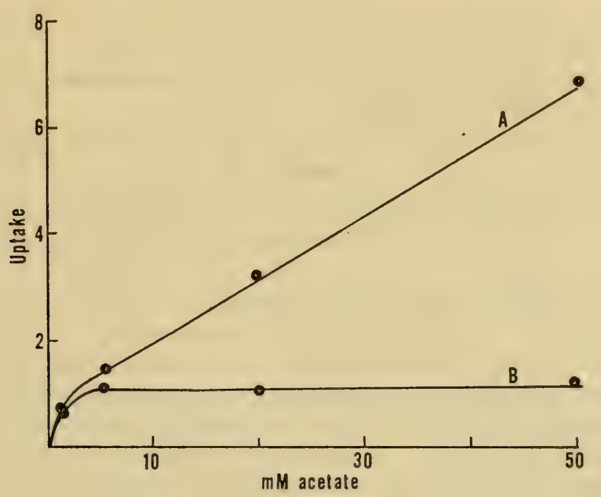

FIGURE 1. The uptake of ^{14}C-acetate as a function of acetate concentration. Curve A is uncorrected uptake. Curve B is uptake corrected by subtracting diffusion, calculated from the linear portion of Curve A. Each point is a mean of 4 determinations.

RESULTS

The relationship between acetate uptake and concentration is shown in Figure 1. The data indicate that the uptake system is not fully saturated even at the highest concentrations tested. At concentrations from 5 to 50 m*M* the uptake rate increases linearly with increase in concentrations, whereas below approximately 2 m*M*, acetate uptake is not a linear function of concentration. This suggests that a mediated transport mechanism operates during the uptake of acetate but, at high acetate concentrations, mediated transport is masked by a diffusion component. When it is assumed that at high concentrations of acetate, mediated uptake mechanisms are saturated, a diffusion rate of 0.12 μmoles/g./min. per unit m*M* increase in acetate concentration is calculable from the data in Figure 1, curve A. If the data are corrected for a diffusion component of uptake, a typical adsorption isotherm is obtained (Fig. 1, curve B). However, the data do not rule out conclusively the

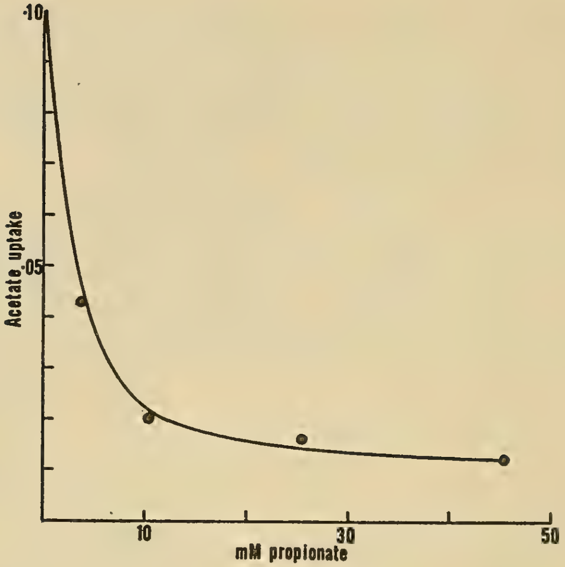

FIGURE 2. The effect of increasing concentrations of propionate on the uptake of acetate (0.1 m*M*). Each point is a mean of 8 determinations.

possibility that a second system for the mediated transport of acetate is involved, since a second system, saturable at very high concentrations, might appear to yield a linear relationship over the concentration range investigated during the present study. The latter probability is diminished, however, by the observation that propionate does not appear to affect the second component of acetate absorption (Fig. 2) ; the residual absorption of 0.1 m*M* acetate in the presence of 50 m*M* propionate is essentially equal to the diffusion value calculated from the linear portion of the uptake curve shown in Figure 1.

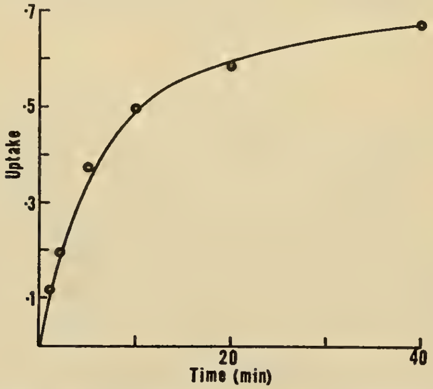

FIGURE 3. Ethanol-extractable ^{41}C, expressed as μmoles ^{14}C-acetate/g, after incubation in 0.1 m*M* ^{14}C-acetate. Each point is a mean of 4 determinations.

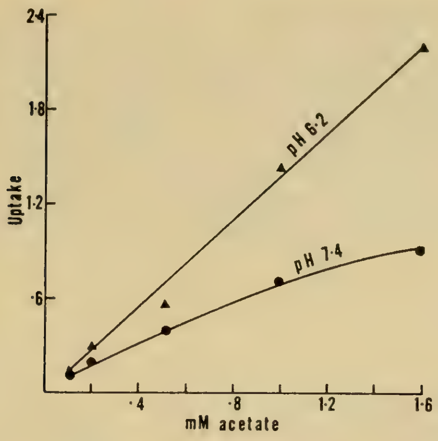

FIGURE 4. The uptake of ^{14}C-acetate at pH 6.2 and 7.4 as a function of acetate concentration. Each point is a mean of 8 determinations.

Analysis of the data on acetate uptake in terms of Michaelis-Menten kinetics allowed an evaluation of K_t (equivalent to Michaelis' constant) and V_{max} (extrapolated maximum velocity). In the concentration range 0.1 to 1.0 m*M* acetate, K_t and V_{max} were 1.13 m*M* and 1.25 µmoles/g./min., respectively, when the data were not corrected for diffusion. After correction, K_t was 0.92 m*M* and V_{max} was 0.91.

The uptake of sodium acetate with respect to time is shown in Figure 3. Following 40 minutes incubation in a substrate concentration of 0.1 m*M* the amount of radioactive material extracted from the worm indicated an internal acetate concentration in the worm water of 0.175 m*M*. Preliminary chromatography indicated that about 60% of the radioactivity extracted was unaltered acetate, but further study would be required to determine more precisely the proportion of the radioactivity representing metabolically unaltered acetate. However, it may be suggested that mediated acetate uptake is a facilitated diffusion rather than active transport.

TABLE I

Effects of pH on acetate uptake and on propionate inhibition of acetate uptake. Acetate concentration 0.1 mM; propionate concentration 4 mM

pH	Uptake (mµmoles/g./min. ± S.E.)		% Inhibition
	Acetate alone	Acetate + propionate	
6.1	161 ± 10.4	134 ± 5.0	17
6.4	147 ± 9.4	102 ± 5.1	31
6.7	139 ± 9.0	64 ± 2.3	54
7.0	135 ± 14.2	53 ± 1.4	61
7.5	129 ± 7.6	39 ± 1.8	70

Influence of pH on acetate absorption

Worms were pre-incubated for 30 min. in KRT at pH 7.4 and then incubated for 1 min. in 0.1 m*M* acetate over a pH range of 6.2–8.0. Radioactivity in incubation solutions was assayed at the end of the experiments in order to determine whether any acetate was lost at low pH; no loss was detectable over the time period of the experiment. Acetate uptake increased as a function of decreasing pH and propionate inhibition of acetate decreased with decreasing pH (Table I). The uptake of acetate at pH 6.2 and 7.4 was studied over a substrate concentration range of 0.1–1.6 m*M* and the results are shown in Figure 4. In contrast to the results obtained at pH 7.4, the uptake of acetate at pH 6.2 was a linear function of acetate concentration.

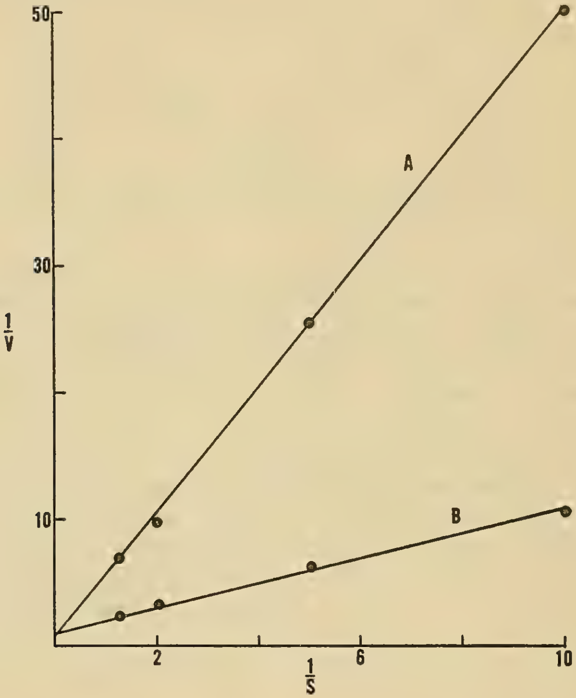

FIGURE 5. The effect of 4 m*M* propionate on the uptake of ^{14}C-acetate in one-minute incubations. A = inhibited slope; B = uninhibited slope; V = uptake, and S = m*M* acetate. Each point is a mean of 12 determinations.

Effects of other compounds

Effects on acetate uptake of the addition of a variety of compounds were examined. The results of these experiments are shown in Table II. Of the compounds tested, only short chain volatile fatty acids produced a significant effect on acetate uptake.

Acetate uptake at various concentrations in the presence or absence of the individual inhibitory fatty acids of Table II was examined by the Lineweaver-Burke method (1934). A typical double reciprocal plot, that of the effects of propionate on the uptake of acetate, is shown in Figure 5. The approximately common intercept of the slopes of both the inhibited and non-inhibited plots indicates that the inhibition was competitive in nature. Using the K_t and V_{max} values determined experimentally for acetate uptake, the inhibition constants (K_i) for the activity of other fatty acids were determined by application of the equation:

$$\text{Slope} = \frac{K_t}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$

TABLE II

Effects of various compounds on acetate uptake in 1 minute. Acetate concentration, 0.1 mM; inhibitor concentration, 4.0 mM

Addition	Uptake (mμmoles/g./min. ± S.E.)	% Inhibition
none	105 ± 6.6	—
2,4-dinitrophenol	102 ± 8.4	0
phlorizin	104 ± 9.2	0
ouabain	107 ± 6.3	0
adenine	101 ± 12.1	0
uracil	102 ± 9.3	0
galactose	96 ± 7.2	0
glucose	107 ± 6.1	0
glycerol	96 ± 4.5	0
alanine	119 ± 11.0	0
phenylalanine	120 ± 10.0	0
arginine	97 ± 6.9	0
glutamic acid	93 ± 7.5	0
leucine	104 ± 6.9	0
lysine	110 ± 10.7	0
betaine	103 ± 8.9	0
sarcosine	121 ± 9.0	0
lactate	91 ± 14.9	0
succinate	124 ± 5.9	0
formate	81 ± 2.3	23
propionate	33 ± 1.8	69
butyrate	53 ± 2.9	50
valerate	63 ± 2.9	40
octanoate	63 ± 1.4	40

Values for the inhibitor constants of the fatty acids studied are: formate 6.9; propionate 1.25; butyrate 3.6; valerate 5.0; and octanoate 5.1.

To determine whether the inhibitions were fully or partially competitive, the uptake of acetate at a concentration of 0.1 mM was studied in the presence of inhibitor concentration of 1 to 10 mM; the data were then plotted after the method of Dixon (1953). Only formate and acetate were found to be fully competitive inhibitors of acetate uptake.

By an extension of the Michaelis-Menten treatment of enzyme kinetics, Read,

TABLE III

Observed and predicted effects of a mixture of fatty acids on 1-minute acetate uptake. Inhibitors present were sodium propionate (2 mM), sodium butyrate (2 mM), and sodium formate (4 mM)

Acetate concentration (mM)	Uptake mμmoles/g./min. ± S.E.	
	Observed	Predicted
0.1	28 ± 0.6	25
0.2	45 ± 1.7	50
0.4	110 ± 10.2	92
0.6	154 ± 8.6	131

Rothman and Simmons (1963) derived an equation predicting the inhibitory effects of a mixture of amino acids on the uptake of a single amino acid. During the present investigation the effects of a mixture of fatty acids on the uptake of sodium acetate were investigated. Inhibitory effects of a mixture of fatty acids consisting of propionate (2 mM), butyrate (2 mM) and formate (4 mM) on the uptake of acetate at several concentrations are shown in Table III in which observed values are compared to those predicted from the equation:

$$V = \frac{V_{max}}{\frac{K_t}{S} + 1 + \frac{(K_t)\ (I)}{(K_i)\ (S)} + \frac{(K_t)\ (I')}{(K_i')\ (S)} + \frac{(K_t)\ (I'')}{(K_i'')\ (S)}}$$

where V_{max}, K_t, and S are values determined for acetate; K_i, K_i', and K_i'' are independently determined inhibition constants for formate, propionate, and butyrate; and I, I′, and I″ are concentrations of the inhibitors.

Uptake of butyrate 1-^{14}C

A limited number of observations were made concerning the relationship between butyrate uptake and concentration between 0.1 and 1.6 mM. Over this concentration range the amount of butyrate absorbed by the tapeworm did not increase

TABLE IV

Relative effects of various fatty acids as inhibitors of acetate and butyrate uptake in 1 minute

Inhibitor	Percentage inhibition	
	^{14}C-acetate	^{14}C-butyrate
Formate	22.9	8.2
Acetate	32.4	16.1
Propionate	69	40.1
Butyrate	50	—
Valerate	40	27.0
Octanoate	40	21.7

linearly with concentration increase and, by methods similar to those outlined above, the K_t and V_{max} values for the butyrate system were 1.5 and 2.5, respectively.

Short chain fatty acids were found to inhibit butyrate uptake and the relative effects of the various inhibitors on both butyrate and acetate uptake are shown in Table IV. The similarity of the relative activity of these several fatty acids as inhibitors of acetate or butyrate uptake suggests similar relative affinities of these inhibitors for the acetate and butyrate transport systems. The uptake of butyrate at several concentrations was determined in the presence and absence of acetate. A Lineweaver-Burke plot of the data so obtained showed the inhibition produced by acetate to be competitive in character and a K_i value of 5.4 for acetate as an inhibitor of butyrate uptake was calculated.

Discussion

It is known that fatty acids enter a number of vertebrate tissues (Fredrickson and Gordon, 1958; Johnston, 1959; Isselbacher, 1965; Quastel, 1965; Hungate, 1966; and others). Quastel (1965) reported that brain tissue accumulated acetate. Transport of acetate, propionate, butyrate, valerate, and hexanoate against a concentration difference has been reported to occur in the small intestine of the rat (Smyth and Taylor, 1958; Barry and Smyth, 1960), although in 60-min. incubations, the concentration ratios reported by the latter workers were always less than 2.0 and the chemical methods used to evaluate the acids were not at all specific (titration after steam distillation).

There is little published information on the kinetics of fatty acid transport. The data of Barry and Smyth (1960) do not permit a conclusion as to whether there is a diffusion component in the absorption of fatty acids by the rat intestine. These workers assumed Michaelis-Menten kinetics and calculated a K_m of 45 m*M*. This may be a spurious value since the data do not show saturation kinetics and may represent a combination of mechanisms for acetate absorption.

The present experiments have shown that a significant proportion of acetate absorption by *Hymenolepis* occurs by a mediated process. At concentrations above 2 m*M*, diffusion appears to be a significant component of acetate uptake. In this dual mode of absorption, the uptake of acetate resembles the uptake of uracil by *Hymenolepis* (MacInnis *et al.,* 1965). Since acetate transport is not affected by a variety of organic compounds, other than fatty acids, it may be concluded that acetate transport in *H. diminuta* occurs through a mechanism with fatty acid specificity. The competitive inhibition of acetate uptake by other short chain fatty acids is further evidence for a specific mediated process and suggests that there is a common transport system for these compounds. Indeed, the similarity in the relative inhibition of acetate or butyrate uptake by the other fatty acids is strong evidence for common transport mechanisms for these two compounds, but more extensive experimentation is required to ascertain whether mediated transport of formate, propionate, valerate, heptanoate, and octanoate occurs only through the acetate-butyrate system.

The effects of pH on acetate uptake suggest that the undissociated form enters more rapidly by diffusion, perhaps because of higher solubility in membrane lipid. The data further suggest that mediated transport mainly involves the dissociated form of acetate. The effect of pH on the propionate inhibition of acetate is also consistent with the above interpretations.

Ginger and Fairbairn (1966b) remarked that during a 2-hour period the rate of absorption of acetate by *H. diminuta* was linear. Estimation of the concentration of acetate present in Ginger and Fairbairn's experiment (using 2.5 μc. acetate, with specific activity of 2.05 μc./m*M,* in a volume of 10 ml.) indicates a substrate concentration of about 0.121 m*M*. At this concentration, the absorption of acetate during a 2-hour period should be non-linear with time. However, Ginger and Fairbairn's measurements were of the incorporation of acetate into lipid which may not be a measure of absorption. For example, the incorporation of ^{14}C-labeled amino acids into protein in *Hymenolepis* or *Calliobothrium* is linear with time whereas absorption follows first order kinetics (Harris and Read, in preparation; Fisher *et al.,* in preparation). Care must be taken to differentiate absorption from incorporation into other tissue components. It is apparent that in many cases the rate of absorption does not limit the rate of incorporation of a substance. Or, putting it in different terms, the concentrations required to saturate incorporation systems may be quite low when compared with concentrations required to saturate absorption systems. Similarly, the use of the term "uptake" may create confusion. In recent studies on *Mycoplasma,* Rottem and Razin (1967) reported that acetate "uptake" was decreased by butyrate and propionate. These authors speculated that the effect involved inhibition of acetokinase activity, but it is not possible to determine from the data available whether absorption mechanisms or intracellular enzyme reactions are involved. It would be helpful if the term "uptake" were restricted to denote the movement of solute from an extracellular to an intracellular location.

The competition between short chain fatty acids in their absorption by this worm should be considered in terms of (1) the probability that the quality and relative quantities of fatty acids in the small gut are relatively constant and, to some extent, independent of the fatty acids ingested by the host, and (2) the apparent inability of the worm to carry out *de novo* biosynthesis of higher fatty acids (Ginger and Fairbairn, 1966b; Jacobsen and Fairbairn, 1967).

Harrington (1965) showed that the components of *H. diminuta* lipids are quantitatively altered when the worm is reared in hamsters rather than rats. A clue to the basis of such alterations is found in the evidence that a considerable portion of the fatty acids in the lumen of the small intestine are derived from the host (Ginger and Fairbairn, 1966b).

When this is coupled with the finding that *H. diminuta* appears to be quite limited in its ability to synthesize fatty acids, the worms being limited to reactions resulting in chain lengthening (Ginger and Fairbairn, 1966; Jacobsen and Fairbairn, 1967), specific characteristics of fatty acids of host origin should be reflected in the lipid composition of the worm. Tentative acceptance of this interpretation leads to the conclusion that the transport of fatty acids into the tissues of the worm may be of considerable importance in determining the lipid composition and, in this context, the competitions between fatty acids observed in the present study assume more meaning.

Acetate has been reported to be an end-product of carbohydrate metabolism in *H. diminuta* and to be excreted into the external medium by the worm (Fairbairn *et al.,* 1961). It might be argued that acetate absorption is of no physiological significance since the worm produces the compound in considerable quantity. However, it has been shown that labeled acetate in the external medium enters the tissues and is incorporated into tissue lipids (Ginger and Fairbairn, 1966b; Jacobsen and Fairbairn, 1967). The latter authors remarked that the incorporation of ^{14}C-acetate occurred at a much lower rate than the incorporation of higher fatty acids. This might be attributable to dilution of the labeled acetate by the unlabeled acetate originating from carbohydrate metabolism. On the other hand, Ginger and Fairbairn (1966b) observed that only small amounts of ^{14}C previously incorporated into glycogen appeared in lipid during a prolonged incubation in which most of the glycogen was metabolized. This does not appear to be completely consistent with the view that significant portions of the acetate produced endogenously are incorporated into lipid. Further investigation of the relative significance of acetate from metabolic and external sources would be desirable. The present authors can offer no explanation for Daugherty's (1957) report that significant amounts of acetate do not enter the tissues of *H. diminuta* in 15-min. incubations at 37° C.

It is tacitly recognized that the absorption of fatty acids by the worm living in a host may involve important modifications not duplicated in the present experiments. There is evidence that, in the digestive tract, fatty acids, monoglycerides, and conjugated bile salts are present as mixed micelles (Hofmann and Borgström, 1962). This may be of great significance in the absorption of higher fatty acids which are relatively insoluble in water, even at soaps. Thus, while bile may serve as a source of fatty acids (Baxter, 1966), it also contributes bile salts which may be of significance in fatty acid absorption. An investigation of the effects of bile salts on absorption of fatty acids by *Hymenolepis* will be the subject of a subsequent portion of this study. The effects of pH on fatty acid uptake suggest that the antero-posterior pH gradient in the small intestine of the host may be significant in determining the rate of fatty acid uptake and the relative roles of mediated transport and diffusion.

Of a number tested, the only compounds producing competitive or partially competitive inhibition of acetate or butyrate uptake were other volatile fatty acids. This supports the view that the mediated transport of these fatty acids occurs through mechanisms showing some specificity. As a matter of fact, four types of mediated transport showing chemical group specificity are now known in *Hymenolepis diminuta.* Group A includes mechanisms for the mediated transport of amino acids (Read, Rothman and Simmons, 1963); Group B includes mechanisms for the mediated transport of monosaccharide sugars (Phifer, 1960a, b; Read, 1961); Group C includes mechanisms for the transport of purine and pyrimidine bases (MacInnis, Fisher and Read, 1965); and Group D, identified in the present work, includes mechanisms for transport of short chain fatty acids. At higher concentrations, considerable quantities of the compounds of Groups C and D enter the worm tissues by diffusion. Preliminary observations suggest that higher fatty acids, such as palmitic, may be absorbed by *H. diminuta* through mechanisms which are independent of those involved in the absorption of the short chain fatty acids. Transport of higher fatty acids is being examined in some detail.

The technical assistance of Miss Linda J. Rogers is gratefully acknowledged.

Summary

1. At low substrate concentrations (below approximately 2 mM) *Hymenolepis diminuta* absorbs acetate by a mediated process; at high substrate concentrations the main mode of entry is diffusion.

2. Hydrogen ion concentration affects acetate uptake. At low pH the rate is increased and there is a larger diffusion component. Inhibition of acetate uptake by propionate is depressed at low pH.

3. Acetate uptake is inhibited by other short chain fatty acids but is unaffected by a variety of other compounds. The kinetics of the inhibitions were examined and inhibitions were found to be partially competitive in the case of propionate, butyrate, valerate and octanoate and fully competitive in the case of formate. Butyrate uptake is also inhibited by these compounds.

4. The data are discussed in terms of transport of fatty acids, lipid metabolism in cestodes and possible significance in the ecology of the organism.

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THE EFFECTS OF HYDROCORTISONE ON THE BLOOD OF TADPOLES AND FROGS, RANA CATESBEIANA

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In his 1957 monograph, Chester Jones stated, "Little information is available from the results of the injection of adrenocortical steroids, used in mammalian work, into Amphibia" (pp. 153–154). Since that time, various observational and experimental attacks on the anterior pituitary-adrenocortical system of the Amphibia have increased our understanding of the adrenal cortex, its role and its control in this group of vertebrates. Aldosterone and corticosterone have been identified as the major cortical steroids of the anurans (Carstensen, Burgers and Li, 1961; Hanke and Weber, 1964; Pesonen and Rapola, 1962; and Phillips and Bellamy, 1963), and recently, Piper and de Roos (1967) have found that both these corticoids have negative feedback influence on the pituitary's secretion of ACTH in *Rana catesbeiana.* Hydrocortisone, an obvious product of the adrenal cortex of mammals, seems to be of lesser significance in the amphibians, although it has been found in cortical extracts or in excretory products of representatives of several genera of this group (Chester Jones, Phillips and Holmes, 1959; Dale, 1962; and Pesonen and Rapola, 1962). Further, studies in our laboratory have established that responses to injections of this hormone do occur in *Rana pipiens* (Bennett and Alspaugh, 1964; and Bennett and Newell, 1965). Changes in the distribution of leucocytes, similar to those seen in mammals and reported originally by Dalton and Selye (1938), were found as early as 12 hours after and as late as 144 hours after the treatment of frogs with hydrocortisone acetate. Of what significance these reactions are in the normal economy of *R. pipiens* is not known. The possible stress imposed by dehydration did not effect lymphopenia and neutrophilia in these frogs as did the injection of hydrocortisone (unpublished observations).

Among the many other questions we have regarding the role of hydrocortisone in Amphibia are these: does this steroid evoke changes in the blood picture of species other than *R. pipiens;* does it have effects on the distribution of white blood cells in immature as well as mature anurans? These are the questions which this study attempted to answer.

Materials and Methods

The tadpoles, *R. catesbeiana,* were obtained from a supplier in North Carolina in October, 1967. They were kept in battery jars of pond water at a temperature which ranged between 9.5° and 10.5° C. Fifty tadpoles were studied. The experimental animals, 25, were injected with 0.5 cc. of a 0.2% suspension of hydrocortisone acetate (Nutritional Biochemical Corporation) in distilled water, and the controls, 25, were injected with the same amount of distilled water. The injections were made into the musculature of the tail in all cases. Forty-eight hours after injection, blood was obtained from the tail; smears were stained with Wright's blood stain. Differential counts of 100 leucocytes were made for each animal.

Frogs, *R. catesbeiana*, were sent from a supplier in Wisconsin from October through December 1967. These animals were kept in individual battery jars containing about 2 cm. of tap water in a room in which the temperature varied between 9.5° and 10.5° C. The 24 experimental animals received 1 cc. of a 0.2% suspension of hydrocortisone acetate in distilled water while 24 controls were injected with 1 cc. of distilled water. The injections were made under the skin of the ventrolateral surface of the posterior trunk. Again, blood samples were withdrawn 48 hours after treatment. Smears were stained with Wright's stain, and differential counts of 100 white cells were made for each frog.

RESULTS AND DISCUSSION

The average per cent distributions of neutrophils and lymphocytes for the control and experimental animals are given in Table I. Also included in this table are the Student's "t" values for the differences between the two types of blood cells for the controls and experimentals. The difference between the number of lymphocytes in the control and experimental tadpoles was found to be significant ($P < 0.025$), as was the difference between the number of neutrophils in these two groups of animals ($P < 0.01$). The number of lymphocytes in the control frogs was much greater than in the experimentals, and again, the difference was significant ($P < 0.005$). The difference between the counts of neutrophils in these two groups of frogs was also significant ($P < 0.01$).

TABLE I

The average per cent distribution of lymphocytes and neutrophils and the Student's "t" values for the differences between counts of the two types of cells for control and experimental tadpoles and frogs

Animals	% Lymphocytes	"t"	% Neutrophils	"t"
Experimental tadpoles	30.08		49.82	
Control tadpoles	58.12	2.07	21.48	2.44
Experimental frogs	17.50		70.71	
Control frogs	57.46	3.10	33.46	2.23

Thus, it is evident that both the immature and mature forms of *R. catesbeiana* responded to the hydrocortisone as do mature *R. pipiens*. The directions of the responses, *i.e.*, an increase in neutrophils and a decrease in lymphocytes, were also the same in the three groups of animals. However, the average intensity of the reactions varied. In the experimental tadpoles, the neutrophils increased 131% over those of the controls while the lymphocytes decreased 49%. For the experimental frogs, *R. catesbeiana*, the increase in neutrophils was 111%, and the decrease in lymphocytes was 69%. Forty-eight hours after the injection of the same amount of hydrocortisone as was used in the present study, the number of neutrophils was

95% greater and the number of lymphocytes was 41% lesser in mature, experimental *R. pipiens* than in their controls (Bennett and Newell, 1965).

The significant decreases in monocytes, eosinophils and basophils which were observed 72 and 144 hours after administration of comparable doses of hydrocortisone to *R. pipiens* (Bennett and Alspaugh, 1964) were not seen in the blood of *R. catesbeiana* or of adult *R. pipiens* 48 hours after injection of the steroid (Bennett and Newell, 1965). The distribution of these rarer leucocytes may change only after more than 48 hours of exposure to hydrocortisone. However, the distribution of leucocytes in normal frogs varies widely; thus valid generalizations about even the *typical* white blood cell picture are difficult to state (Schermer, 1967). The differences in intensity of neutrophilia and lymphopenia reported here, as well as the differences between the counts of monocytes, basophils and eosinophils of experimental *R. catesbeiana* and *R. pipiens* at 48 hours and those at 72 and 144 hours after treatment, are in reality only slight and are probably not of biological significance.

The increase in neutrophils and the decrease in lymphocytes in the two species of frogs are certainly significant, and are of interest, especially since they parallel those well known in mammals in which titers of hydrocortisone have been increased by physiological stress or injection. The review of Selye (1950) continues to provide us with details of these changes in mammals. The exact manner in which some of the adrenal steroids effect the responses observed in the blood continues to be problematical. Also not fully understood is the role of the adrenocortical steroids in the immature and/or metamorphosing anuran. Frieden and Naile (1955) reported that hydrocortisone accelerated metamorphosis of thyroxin-treated *R. pipiens* and *R. hechsheri.* The histochemical studies of Rapola (1963) on *Xenopus laevis* also implicate adrenal steroids in metamorphic phenomena. Our work proves only that immature *R. catesbeiana,* as well as mature frogs of this species, do have the system which causes the blood picture of the animals to respond to hydrocortisone as does mature *R. pipiens.* What the mechanism responsible for these changes is; whether it operates in response to intrinsic steroids; whether it is of biological value to these amphibians remain unknown.

Summary and Conclusions

1. Differential counts of the leucocytes of tadpoles and frogs, *R. catesbeiana,* were made 48 hours after the injection of hydrocortisone acetate (experimentals) or distilled water (controls).

2. Neutrophilia and lymphopenia were evident in both the immature and mature experimental animals but not in the controls.

3. These changes in the distribution of white blood cells resemble those known in mammals and in *R. pipiens.*

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COLONY GROWTH AND PATTERN IN THE TWO–TENTACLED HYDROID, PROBOSCIDACTYLA FLAVICIRRATA

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Proboscidactyla flavicirrata is a species of hydroid whose colony is intimately patterned around its commensal host, a sabellid worm. The hydroid colony covers the distal end of the leathery worm tube, and the polyps interact with the worm during feeding (Uchida and Okuda, 1941; Hand and Hendrickson, 1950). The colony pattern has been described by several workers (Hand, 1954; Brinckmann and Vannucci, 1965) but there is virtually nothing known about the colony development.

The observations presented in this paper indicate that the colony growth of *Proboscidactyla flavicirrata* is dynamic and approaches a steady condition. The colony expands as the worm elongates its tube distally. The colony degenerates in regions distant from the tip. The various axial regions of the stolon mat therefore represent successive ages of tissue. This growth pattern results largely from the unusual ability of the feeding polyps to move along the tube, keeping pace with tube elongation and generating a stolon system behind them as they advance.

METHODS AND MATERIALS

Colonies of *Proboscidactyla flavicirrata* (Brandt) were collected during the summer months from large sabellid tubes (over 5 mm. in diameter) growing on the town floats at Friday Harbor, Washington. During the winter months colonies were kindly collected by Dr. Robert Fernald. Observations on colony and polyp progressions were made on material kept in a running sea water table, with the polyps fed every two days on sabellid eggs.

Polyps were explanted on microscope slides by placing them, with a small portion of attached stolon, upright on the surface. In grafting experiments, polyp segments were isolated with a scalpel and strung together on glass fibers to heal for three hours.

The terms *distal* and *proximal* refer to portions of the worm tube corresponding to the worm's anterior and posterior directions, respectively; the open end of the worm tube is distal. The term forward is used in reference to gastrozooid orientation, forward being that direction in which the mouth and tentacles point.

RESULTS

Description of the colony

A *Proboscidactyla* colony is composed of a stolon network and three polyp types: gastrozooid, gonozooid (blastostyle), and dactylozooid. Polyps and stolons are patterned axially along the distal portion of a sabellid worm tube.

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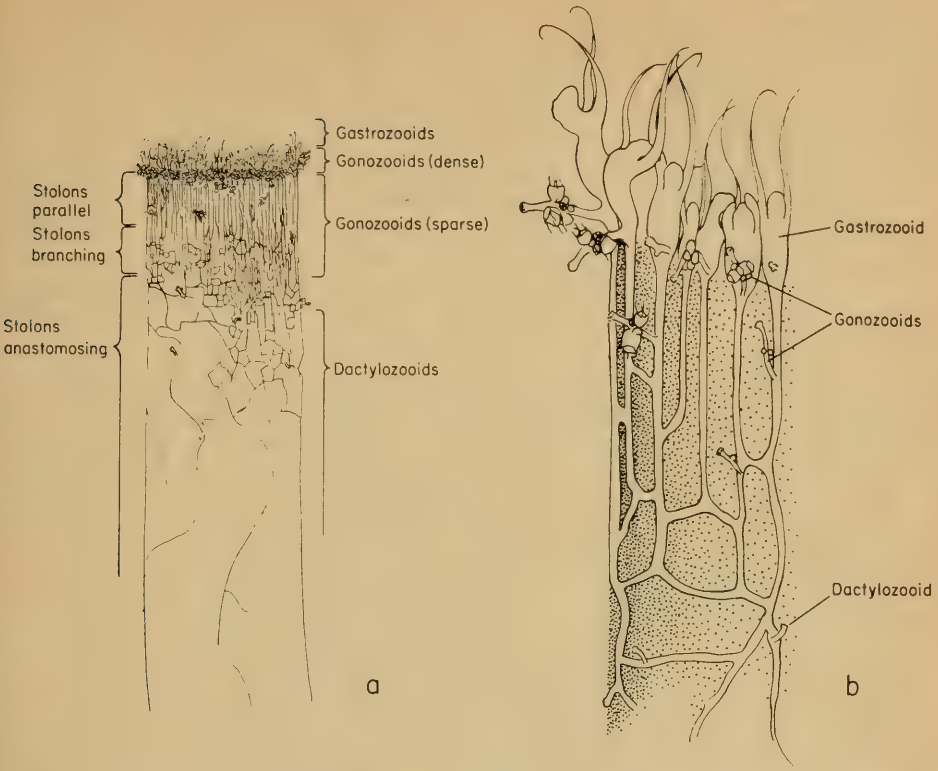

FIGURE 1. *Proboscidactyla flavicirrata* colony pattern. 1a: Axial colonial regions along worm tube. 1b: Polyp insertion and stolon pattern (longitudinal axis greatly shortened).

The gastrozooids are located almost exclusively in a whorl at the rim of the tube (Fig. 1). Gastrozooids are bilateral in structure (Figs. 1b, 3). The curved hypostome inserts vertically on the tip of the arching body column and points in the same direction as the body column arches (Fig. 3), so that the mouth is directed "forward." The two tentacles arising at the base of the hypostome are also directed forward, and a battery of nematocysts is located on the upper side of the hypostome. At the base of the polyp, protruding forward, is a small "foot" (Fig. 3) (see Campbell, 1968a), which may be homologous with the stolon tip of other hydroids. The single stolon connected to each gastrozooid inserts at the base of the polyp "back" (Fig. 3). The stolons (Fig. 1) leading back from the gastrozooids regularly run parallel to the tube axis and alongside adjacent stolons for several millimeters. Further from the tube tip, the stolons become thinner and show increasing branching, thus disrupting the strictly parallel arrangement. In the proximal portions they form an irregular network which becomes indiscernible farther back along the tube.

The gonozooid (Fig. 1), a long column capped by a nematocyst cluster, bears a bouquet of medusa buds midway along its length. Most gonozooids insert on the stolon immediately behind a gastrozooid or on a gastrozooid column. (Almost all

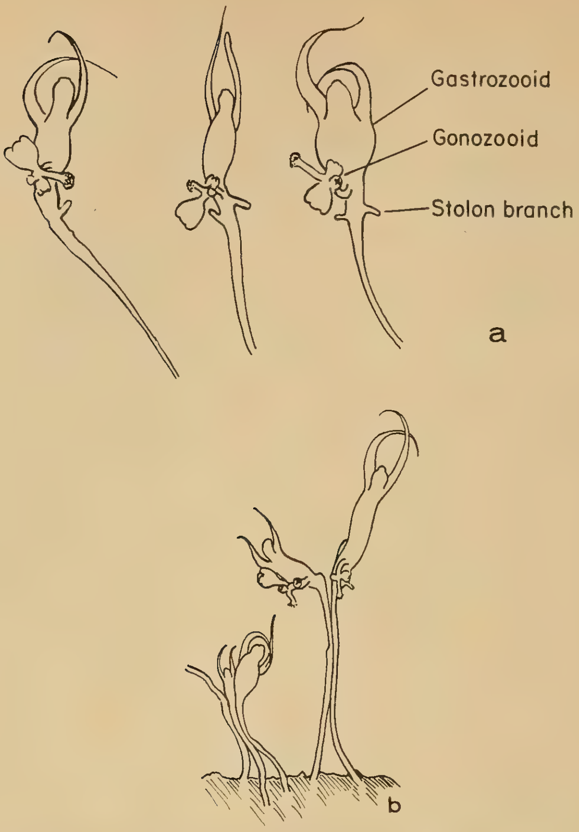

FIGURE 2. Gastrozooids, with gonozooids, migrating along glass tube, 24 hours after colony was streched over the glass. Tracings from photographs. 2a: Three polyps which have stopped migrating, with stolon branches developing behind. These branches resemble those giving rise to new gastrozooids and to stolon anastomosis. 2b: Several polyps showing tendency to follow stolons. Shaded portion represents the edge of the worm tube.

large gastrozooids have such an associated gonozooid.) However, a few gonozooids are scattered sparsely over the distal half of the colony, inserted at random positions on the stolon network.

The dactylozooids (Hirai, 1960) are short, straight or slightly curved polyps, each crowned by a nematocyst bundle. They are scattered over the proximal region of the colony. Uchida and Okuda (1941) have termed such polyps as minute gonozooids lacking medusa buds. Hand (1954) termed them as young or abortive gonozooids.

Intermediate polyp forms may be found between the territories of gonozooids and dactylozooids. These forms are intermediate in length and often bear one or several minute or abortive medusa buds.

The *Proboscidactyla* colony organization is thus predominantly axiate along the tube, with several regions identifiable in terms of stolon patterns and polyp types.

Observations on colony growth and expansion

A. *Overall pattern*

Some colonies were observed for more than two weeks, during which time they grew in length, accommodating elongation of the worm tubes, but did not change in overall pattern. Furthermore, nearly every one of hundreds of colonies studied throughout the summer and at intervals through the winter showed similar organization regardless of the size of the colony and tube (except for colonies on very small tubes, as discussed below). Apparently the colony is in a steady-state developmental pattern, where continuing growth and expansion do not result in a qualitative change in pattern.

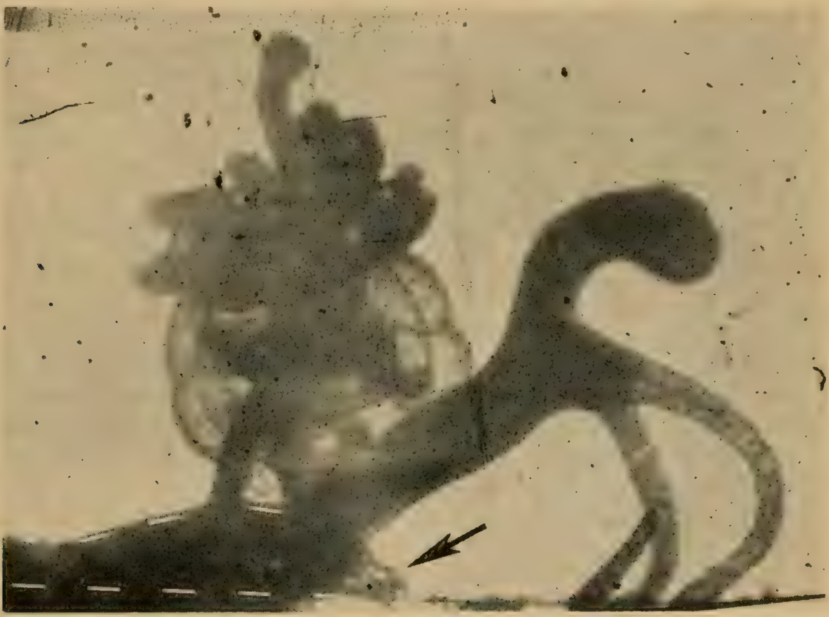

FIGURE 3. Gastrozooid, with gonozooid, migrating on a glass microscope slide. Photograph, taken from the side, was printed from a 16 mm. film. Gastrozooid hypostome is in upper right; two tentacles are at lower right. Arrow points to "foot." The stolon (broken white lines) runs off to the left.

B. *Developmental cycle of polyps*

Gastrozooids arise through modification of a stolon tip at the rim of the worm tube. This stolon forms as a lateral branch from one of the parallel stolons just behind the tube rim. It does not transform into a polyp until after it has extended slightly beyond the tube rim. At this time there is a slight flattening of the tip in a plane tangential to the tube surface. Two tentacle rudiments first become visible as broad lateral wings on this stolon tip. As these elongate, their positions shift towards the "front," where they eventually become spaced about 40°–70° from one another. This shifting is probably due to the more rapid growth of the polyp rudiment on the back surface, rather than to an actual movement of the tentacle rudiments across the column tissue. Tissue above the insertion narrows to form a hypostome. When the polyp rudiment has increased its size by severalfold, it has assumed the typical appearance of an older polyp.

Within a single colony, gastrozooids range from these small young individuals to polyps 4 mm. in length. In some colonies there is evidence of a graded distribution of average polyp size from one side of the tube to the other. No stages of gastrozooid degeneration were consistently found. Thus it appears that gastrozooids arise beside, or intercalated between, existing polyps through transformation of stolon tips, and that the polyps then have a relatively long life during which they grow continuously larger.

Gonozooids arise as small buds on the "backs" of the gastrozooids, about midway up the column. Gonozooid buds were found in no other position on the colony. Almost as soon as they are visible the buds are multilobed. As the stalk elongates, the terminal lobe becomes the nematocyst cap, and grows upwards on its own stalk. The other lobes remain in the midstalk region and become medusa buds. The stalk grows relatively rapidly until it is about the same length as the parent gastrozooid. It also changes its position on the gastrozooid, generally moving to the base or just behind the base of the mother polyp.

Gonozooids scattered behind the terminal polyp whorl have few or no medusa buds.

C. *Migratory behavior of gastrozooids*

Gastrozooids are capable of moving across a substratum in a forward direction. This can be demonstrated by extending the polyps' substratum beyond the tube rim, or by explanting polyps onto an appropriate surface under sea water. Under both conditions the gastrozooids will move forward at rates of up to 15 mm. per day.

A simple way to extend the polyps' substratum is to slip the tube over a glass rod. The elasticity of the tube holds the rim tightly against the glass. After 24 hours, many polyps in such colonies will have moved onto the glass (Figs. 2a, b). A single migrating polyp is shown in Figure 3.

The first visible change in a colony after it is stretched over glass is that the "foot" of each gastrozooid swells and elongates. This is apparent after a few hours. Several lines of evidence indicate that this foot directs the subsequent polyp migration. The zooids move forward although not necessarily in a straight line; when their paths curve the feet are also arched. Polyps tend to be guided by physical edges which only the foot may contact; thus, one polyp will follow the stolon of another (Fig. 2b). Finally, if a polyp is cut off from its base, rotated 90° or 180° and grafted back to the base, movement continues in the direction of stolon and foot orientation, not in the polyp's forward direction.

Gonozooids generally move along with the gastrozooid. But occasionally this close relationship is lost and a gonozooid remains isolated on the stolons while the gastrozooids continue advancing.

Mitotic figures (viewed in stained sections of several dozen migrating polyps and their stolons) are absent from the polyp base, but are abundant along the ectoderm and endoderm of stolons behind moving gastrozooids.

Unfed animals move up to several centimeters during the first 36 hours following isolation, but then stop. If fed, polyps migrate for a longer period, but never for more than three days. It is not clear whether food is limiting in these cases; while isolated gastrozooids do diminish greatly in size during movement, some gastrozooids in intact colonies do not.

After migration has stopped, lateral branches are frequently formed from the stolon just behind the gastrozooid (Fig. 2a).

The forward movement of gastrozooids described above is probably involved in the natural growth of the *Proboscidactyla* colony. When worms were grown for five weeks in filtered sea water, the new increments of tube added by the worm were transparent and thus easily distinguished from the previously existing opaque portions. The polyps continued to remain at the rim of the tube, with the entire whorl of gastrozooids eventually located on the transparent tube. Thus movement of polyps along the worm tube occurs during normal growth.

Occasionally, in natural colonies, individual gastrozooids are found away from the tube rim. These are directed in random orientations, their stolons indicating that they had previously been moving parallel to other polyps, but for some reason wandered from the tube rim.

Deduced colonial growth pattern

The observations outlined above suggest a growth pattern by which the entire colony remains in a steady-state condition. As the worm extends its tube at the distal rim, the gastrozooids advance forward, thereby maintaining their terminal positions. The individual polyps thus remain in a strategic ecological position and probably live for a relatively long time.

As gastrozooids advance, the stolon behind the polyps increases in length. This leads to the typical pattern of parallel stolons encircling the distal portion of the worm tube. The stolon system, therefore, is progressively older towards the proximal end of the tube. Apparently the stolons gradually form lateral branches which fuse, leading to progressive anastomosis at greater distances from the tube rim.

Some lateral branches are present in the distal portion. Generally these branches bend forward and extend toward the tube rim between the parent and adjacent stolon. These young stolons are the source of new gastrozooids, their tips being transformed directly into a polyp. Some control process must eventually limit the rate of new gastrozooid formation; possibly high stolon density inhibits lateral branching.

The gonozooids arise on the lower column of the gastrozooid. They remain attached to, or closely associated with, the mother polyp. Occasionally they fall back, are dissociated from the movements of the gastrozooid, and are left behind in the colony. Actually, they still probably continue moving forward to some extent because stolon growth (judging from mitotic figure distributions) occurs along the length of the unbranched stolon portions. These isolated gonozooids gradually lose their medusa buds and diminish in size, becoming dactylozooids.

This model of colonial growth is shown in Figure 4. The colony consists of an active front, which keeps pace with the advancing tube rim, and a stationary

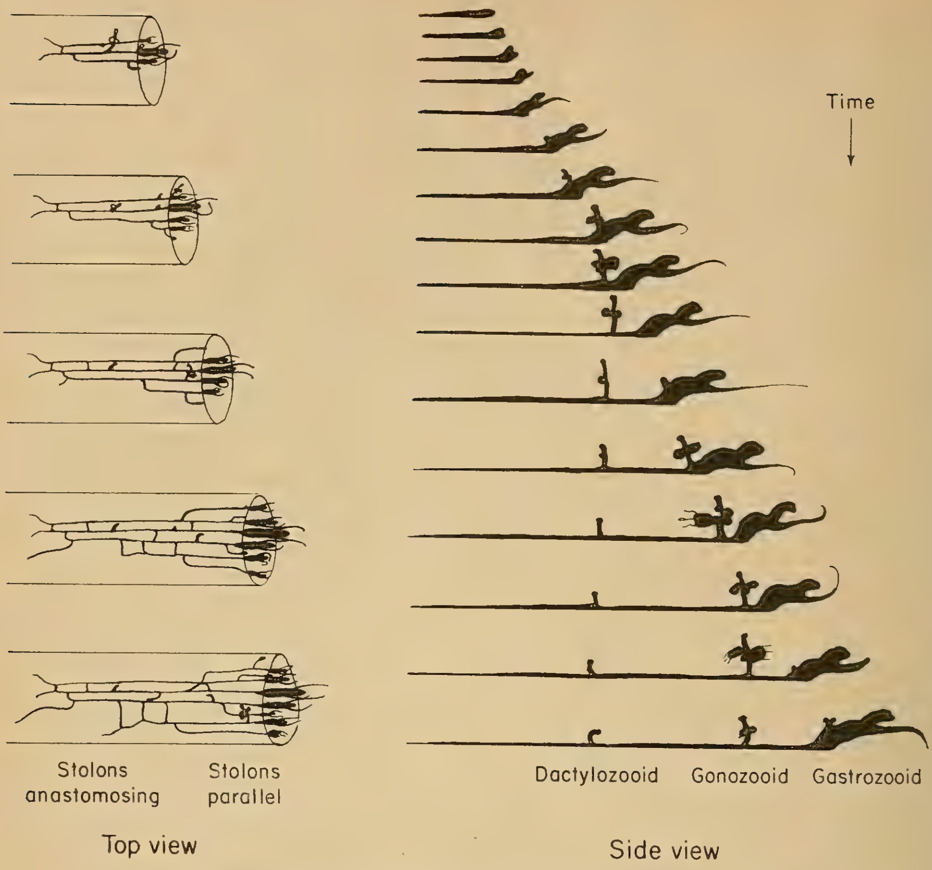

FIGURE 4. Deduced colony progression and development pattern (see text).

network of stolons covering the proximal portion of the worm tube. Individual gastrozooids represent at least relatively permanent components of the active front; they may or may not undergo a gradual replacement cycle. Gonozooids go through a more rapid replacement cycle; individuals occasionally stop advancing with the gastrozooid, stop forming medusa buds, and diminish in size. Each one lost in this manner is replaced by a new one budded from the column of the parent gastrozooid. The stolon system represents the trail of gastrozooids, slowly elaborated by anastomosing.

DISCUSSION

The unique *Proboscidactyla* colonial growth pattern results from the ability of the gastrozooids to move. Colonial succession in other hydroids which have been studied involves extension of the colony boundaries by stolon growth, followed by new polyps arising in the peripheral zones. Older colony regions may die and be resorbed, giving rise to a net displacement of a colony unchanging in size (such as occurs in *Campanularia;* Crowell, 1953) or the older portions may remain active, in which case the colony expands in size (such as in *Podocoryne;* Braverman, 1964). In neither case is there any movement of any portion of the colony relative to the substratum, except for perhaps the terminal portions of the stolons. In *Proboscidactyla,* on the other hand, individual polyps migrate during colony translocation.

This migration pattern shows two points of similarity to the growth patterns of other hydroids. First, the movement of the gastrozooids appears to be similar and perhaps homologous to the elongation of the stolons in other hydroids. According to this view, the "foot" of the *Proboscidactyla* polyp represents the stolon tip; the gastrozooid would therefore be equivalent to a normal polyp which is situated just behind the stolon tip. Spots of vital dye applied near stolon tips in a number of hydroid genera (*Obelia,* Berrill, 1949; *Cordylophora,* Overton, 1963; *Clytia,* Hale, 1964) do move with the tip as does the *Proboscidactyla* gastrozooid (see also Campbell, 1966, 1968b).

Secondly, *Proboscidactyla* polyp movement resembles that of the upright growth in athecate hydroids bearing terminal polyps, where the individual polyp moves away from the colony center through upright elongation. The main difference between the two growth types is the relation of the polyp and stalk to the substratum.

Some descriptions of *Proboscidactyla* colonies do not indicate the presence of a terminal portion where stolons run parallel. These descriptions are of colonies on small worm tubes. I also observed small colonies to lack regular stolon patterns. Probably larger worm tubes elongate more rapidly than small ones. Since stolons do gradually anastomose, one would expect to find parallel cords of stolons during rapid elongation; at these times polyps can advance forward significant distances before stolon branching occurs. This consideration may explain the differences in colony regularity on large and small worm tubes, and the differences in stolon pattern and polyp distributions among related species (see Hand, 1954).

SUMMARY

1. The colony of the hydroid *Proboscidactyla* covers the terminal portions of a sabellid worm tube. The colony is axially patterned. In distal regions stolons run parallel to the form tube axis while in proximal regions they form an anastomosing network. Gastrozooids are situated at the tube rim, gonozooids in a whorl behind gastrozooids, and dactylozooids in the proximal colony regions.

2. Gastrozooids will migrate actively forward when they are not at the edge of a substratum. The gonozooid associated with each gastrozooid generally moves with it.

3. The colony appears to be in a steady-state condition. The whorl of gastrozooids, and associated gonozooids, migrates forward as the tube rim advances due to secretion by the worm. The stolon system is generated behind the advancing gastrozooids. The proximal end of the colony progressively loses its regularity and definition. The axiate patterns of the colony at any moment thus trace out the history of the colony.

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RED CELL MORPHOMETRICS AND VOLUME IN THE CICHLID TELEOST TILAPIA MOSSAMBICA, USING A CHROMIUM-51 LABELING METHOD [1, 2]

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Investigations of blood and blood cell volumes are limited in fishes, but a cross-section of interpretation of blood cell morphology is offered in the reports of Downey (1909), Jordan and Speidel (1930), Catton (1951) and Weinreb (1963). Blood volume studies have been hampered because the methods used to measure the mass of red cells were inadequate for application to fish. Exsanguination has been frequently attempted with limited success (Ronald, MacNab, Stewart and Beaton, 1964). Derrickson and Amberson (1934) used a perfusion technique on the dogfish, replacing blood with isotonic saline. Indirect dye dilution using Evans Blue was applied by Thorson (1961) on several marine and fresh-water species and by Smith and Bell (1964) on pink and sockeye salmon. Ronald *et al.* (1964) have applied fluorescein dye dilution on the Atlantic cod and Conte, Wagner and Harris (1963) used ^{51}Cr, Evans Blue and human serum albumin-^{131}I for blood studies on steelhead trout. The purposes of this investigation were to describe the formed elements and derive the red cell and total blood volumes of *Tilapia mossambica* and to determine the usefulness of ^{51}Cr as a labeling material in this fish.

METHODS AND MATERIALS

The experimental species, *T. mossambica,* was collected from an estuary of the Enchanted Lakes located near Kailua on the windward side of Oahu, Hawaii. The size range was from 150 to 250 mm. (fork length) with weights ranging from 100 to 250 g. Females were of much greater relative abundance than males. The surface temperature of the estuary averaged 26° C. and the salinity varied from 0 to 25 ppt. Captured fish were held in 55-gallon fiberglass aquaria containing flowing fresh water with a steady temperature of 23.3° C.

Experimental animals were anesthesized with MS 222 (Tricaine methanesulfonate) in a 1:10,000 weight-to-volume solution to a deep stage III level, which is characterized by an absence of swimming movements, respiratory activity or response to external stimulation (Klontz, 1964). They were then placed in a restraining device to irrigate the gills with the MS 222 solution using a pump recycling system. The equipment was similar to that used by Smith and Bell (1964) at the Nanaimo, British Columbia, Station of the Fisheries Research

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[1] Part of a thesis submitted to the graduate faculty of the University of Hawaii in partial fulfillment of the requirements for the degree of Master of Science.

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Board of Canada. Because of the unreliability of heart puncture and injection, a technique for cannulation of the ventral aorta was utilized using a variation of a dorsal aorta cannulation employed by Schiffman (1959) and Smith and Bell (1964). The cannula was polyethylene tubing with an inside diameter of 0.025 inch. The tubing was placed over the cut end of a 22-gauge regular bevel needle and filled with a solution of 1% NaCl, 1.2% dextrose and 220 units of heparin per milliliter. The needle was inserted into the skin 4 or 5 mm. to either side of the median sagittal axis of the fish and directly above the ventral aorta. A suitable vessel puncture site was easily selected as the needle and vessel were visible through the membrane of the isthmus. As an attempt to maintain the fish in a constant but an admittedly abnormal environment, the animal was completely immersed in a small blackened aquarium which was supplied with flowing fresh water.

While no tissue erosion was noted around the sutures or puncture sites, hemorrhages developed at the puncture site one to two days after cannulation and after three days, successful sampling could no longer be carried out. It was possible to remove 30 to 50% of the total volume of blood in *Tilapia* without aspiration and no mortalities occurred following repeated injection and blood removal, but it often took the fish 3 or 4 hours to recover to pre-anesthesia levels of activity and appearance.

Smears of fresh, unheparinized blood were used for descriptive study. Smears were fixed in absolute methanol and flooded with Giemsa stain buffered with Sorensen's phosphate buffer to pH 6.8. Red cell dimensions of fixed cells were measured by use of an eyepiece micrometer. Thickness measurements were obtained from living cells in suspension. No differential white cell counts were made. Hematocrits were determined using the micromethod of Snieszko (1960). They were taken from all ventral aorta cannulated fish, serving as controls for blood volume values and were also taken in some fish by severing the tail and exposing the caudal vessels. In each microhematocrit determination three tubes were filled consecutively.

Chromium-51 was supplied as $Na_2{}^{51}CrO_4$ with a specific activity of 20 mc. per milligram. The ^{51}Cr activity of liquid samples, in microhematocrit capillary tubes calibrated to a volume of 0.04 ml., was measured with a crystal scintillation detector. At least two five-minute counts were made on each sample. For labeling, 10 μc. of ^{51}Cr were added to each milliliter of heparinized whole blood in a silicon-coated vial. After this mixture was incubated at room temperature (24° C.) for 75 to 90 minutes, with shaking every 5 minutes, 4 mg. per 10 μc. ^{51}Cr of ascorbic acid were added to reduce free ^{51}Cr. The optimum incubation time was determined from the time labeling activity reached a maximum. At 1 to 10, 20, 30, 45, 50, 60, 75, and 90 minutes after adding ^{51}Cr to a vial of unlabeled blood, 0.01-ml. subsamples of blood were withdrawn and the red cell ^{51}Cr activity was measured. The labeling was inhibited by the addition of an aqueous solution of 0.5 mg. ascorbic acid or by washing the subsample with saline to remove free ^{51}Cr.

The total volume of red cells was calculated from a dilution formula described by Squibb & Co. (1959). About 0.5 to 0.9 ml. of ^{51}Cr-labeled whole blood was injected into the cannula of the donor fish with 0.1 ml. retained as a

TABLE I

The formed elements of T. mossambica blood in circulation

Cell type	Dimensions (microns)					Cell:nuc. ratios (by length)
	Whole cell			Nucleus		
	Length	Width	Thickness	Length	Width	
Small lymphocyte	7.5*		—	6.3*		1.2
Large lymphocyte	12.4*		—	5.8*		2.1
Macrophage	12.9*		—	7.9*		1.6
Thrombocyte	9.1	3.7	—	7.0	3.3	1.3
Erythrocyte**	11 ± 0.7	7.5 ± 0.1	3.0	5.0	3.4	2.2

* Recorded as the greatest dimension through the central axis.
** Numbers per mm.^{3} = 1,549,000 ± 73,000 SD.

reference standard. Serial sampling up to 24 hours post-injection was accomplished by filling capillary tubes directly from the tip of the cannula. Total blood volume was found by dividing the total sample volume of red cells by the respective sample hematocrit and computing an average for all samples.

RESULTS AND DISCUSSION

For comparative purposes the morphological categories of *Tilapia* blood cells were similar to those described by Catton (1951) and Klontz *et al.* (1964),

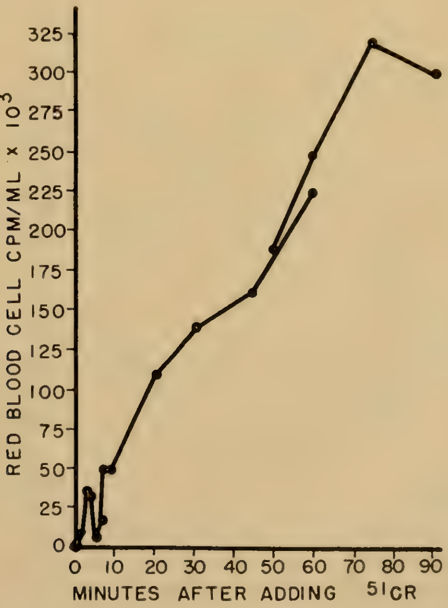

FIGURE 1. Relationship of *in vitro* ^{51}Cr accumulation by *Tilapia mossambica* RBCs as a function of incubation time.

except cells of the granulocytic series were absent in both living and stained preparations. Morphometric and enumeration data are presented in Table I. The average hematocrits were 25.8 ± 4.6% ($N = 29$) for caudal vessel samples and 23.0 ± 3.8% ($N = 9$) for ventral aorta samples obtained prior to blood volume determinations. The nearly 3% difference between the two mean hematocrits was not significant (analysis of variance test; $F = 2.81$, d.f. 1, 36), and probably was a reflection of differences in sampling. Sample hematocrits from ventral aorta cannulations, used for total blood volume determinations, presented a lower average (20.5 ± 4.1%, $N = 7$) than presample hematocrits. This was a result of the decrease in sample size and not of blood loss. There was also a tendency for hematocrits (caudal vessel samples) to decrease with increasing fish weights. For a wide weight range, this may be correlated with activity or oxygen consumption.

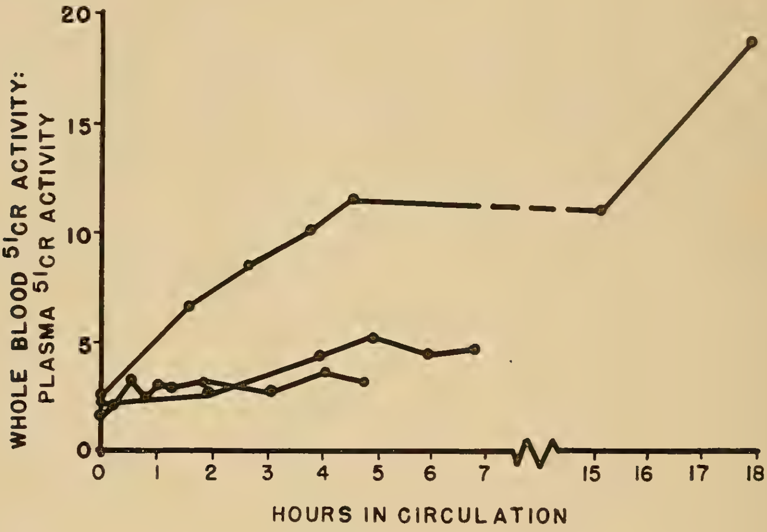

FIGURE 2. Whole blood ^{51}Cr activity: plasma ^{51}Cr activity ratios in three *Tilapia* versus the time of circulation.

The ^{51}Cr uptake curve (Fig. 1) is nearly linear with activity maximizing at 75 to 90 minutes after the addition of ^{51}Cr, although uptake is still rapid at 75 minutes. The dose-dependent effects or possible toxic levels of the ^{51}Cr were not tested. Plasma or unlabeled ^{51}Cr activity levels in *Tilapia* decreased to near background levels 4 to 19 hours after ^{51}Cr infusion in the circulatory system. Initially a large quantity of the radioactivity present in the injection medium does not appear in the red cells. There is a steady decline of plasma activity levels after injection, however, indicating that plasma ^{51}Cr is being rapidly removed from circulation and stored or excreted or both. In all fish samples (serial and single samples from 8 fish) the whole blood activity: plasma activity ratios in the injection medium range from 1.5 to 3.0. After 15 to 19 hours of circulation the values range from 10.2 to 18.8 (Fig. 2). Serial sampling indicates a period of rapid mixing of injected labeled red cells in circulation during the first 10 to 60 minutes, followed by a long period of slow diffusion where fairly constant total red cell volumes or red cell ^{51}Cr activity levels were obtained. Nevertheless, the picture of dilution and circulation in the restrained or confined fish may not be an actual representation of what occurs while it is free-swimming. Since body movements probably aid peripheral circulation, the weak contractions of the heart while the fish is under anesthesia may supply only a limited flow of blood to body elements other than the head, gills, and viscera.

The mean value for the total blood volume was 3.17 ± 0.48 SD ml./100 g. body weight (range = 2.71 to 4.50). The mean value for the total volume of red cells was 0.65 ± 0.14 SD ml./100 g. body weight (range = 0.43 to 0.84, N = 7). A degree of correlation was found among blood volume against weight in seven fish which had moderately constant circulation times (Fig. 3). While the relationship was not significant at the 5% level, there was a definite inverse trend of volume on weight within the weight range tested. This indicates that blood volume in *Tilapia* is not directly proportional with weight as was found in the cod (Ronald *et al.*, 1964) but decreases slightly with increasing weight. The total blood volume of *Tilapia* is consistent with those determinations made for other teleosts (Thorson, 1961; Conte *et al.*, 1963; Ronald *et al.*, 1964).

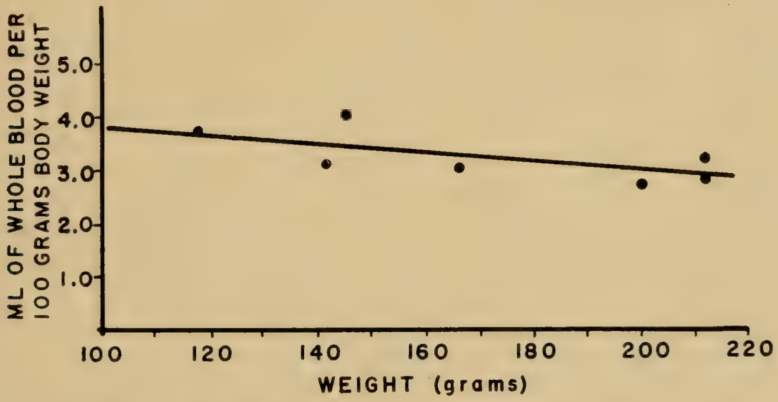

FIGURE 3. Relation of whole blood volume as a percentage of weight (ml. blood/100 g.) on total body weight in *Tilapia mossambica*. Circulation times after the injection of ^{51}Cr labeled RBCs were two to four hours.

SUMMARY

1. Erythrocytes, small and large lymphocytes, macrophages and thrombocytes were identified. No granulocytes were found in stained or living preparations. Hematocrits averaged 25.8 ± 4.6% for caudal vessel samples and 23.0 ± 3.8% for ventral aorta samples. Red cell counts were 1,549,000 ± 73,000 SD per mm^3. Red cells were 11 ± 0.7 μ long, 7.5 ± 0.1 μ wide and 3 μ thick.

2. A technique is described for cannulation of the ventral aorta to allow repetitive sampling or injection while the fish is restrained or confined.

3. The labeling of erythrocytes with ^{51}Cr followed a linear curve with maximum cell incorporation occurring at approximately 90 minutes post-labeling.

4. A long period of slow mixing was noted for injected ^{51}Cr-RBCs after an initial dilution phase of 10 to 60 minutes. The total average total volume of red cells obtained for *Tilapia* as determined by ^{51}Cr dilution was 0.65 ± 0.14 ml./100 g. body weight and the mean total blood volume was 3.17 ± 0.48 ml./100 g. body weight. There was an allometric relationship suggested between TBV as a percentage of body weight and weight. Higher proportional volumes were associated with lighter animals.

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THE LARVAL DEVELOPMENT OF THE COMMENSAL CRAB POLYONYX GIBBESI Haig, 1956 (CRUSTACEA: DECAPODA)[1, 2]

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The larvae of the anomuran crab family Porcellanidae have long been recognized in the plankton by their unique rostral spine (Thompson, 1836). Until recently, however, there was little attempt to rear the larvae in the laboratory under controlled conditions through their complete life cycle. To date, adequate descriptions of the complete larval development are available for species of *Pachycheles* (Kurata, 1964; Knight, 1966; Boschi *et al.*, 1967), *Petrocheles* (Wear, 1965a, 1966), *Petrolisthes* (Gohar and Al Kholy, 1957; Wear, 1964 a, b, 1965b; Greenwood, 1965) *Pisidia* (formerly some *Porcellana* species; Lebour, 1943; Bourdillon-Casanova, 1956), *Porcellana* (Lebour, 1943; Bourdillon-Casanova, 1960) and *Polyonyx* (Knight, 1966).

Polyonyx gibbesi, which is found from Woods Hole, Massachusetts to La Paloma, Uruguay, is a known commensal with the polychaete worm *Chaetopterus variopedatus* (Renier) (see Pearse, 1913; Gray, 1961 and Haig, 1966). Faxon (1879, 1882) briefly described and figured the first and second zoea and the "first stage of the crab" (= megalopa) of *Polyonyx macrocheles* Gibbes (now called *Polyonyx gibbesi* Haig 1956). Faxon's figures and descriptions are unfortunately not sufficiently detailed to allow positive identification of the larvae or comparison with larvae of other species of *Polyonyx*. A second author (MacArthur, 1962, unpublished) also described the larval development of *Polyonyx macrocheles* (sic) but differences between the larvae she studied and mine will require further study before an evaluation can be made.

The purpose of this paper is to illustrate and describe the complete larval development of *Polyonyx gibbesi*. Certain characters are discussed which may enable the larvae of the genus to be identified from the plankton. The relationship between *P. gibbesi* and the Pacific coast form *Polyonyx quadriungulatus* is also discussed.

MATERIALS AND METHODS

Ovigerous female *Polyonyx* were collected from *Chaetopterus* tubes obtained by bucket dredge in the Cape Florida Channel, from an area 3 m. deep and

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[1] Contribution No. 939 from the Institute of Marine Sciences, University of Miami, Florida.

[2] Part of a thesis presented to the Graduate Council of the University of Miami in partial fulfillment of the requirements for the degree of Master of Science. This study was supported in part by Research Grant GM-11244 from the National Institutes of Health, U. S. Public Health Service and a National Science Foundation Institutional Grant through the University of Miami.

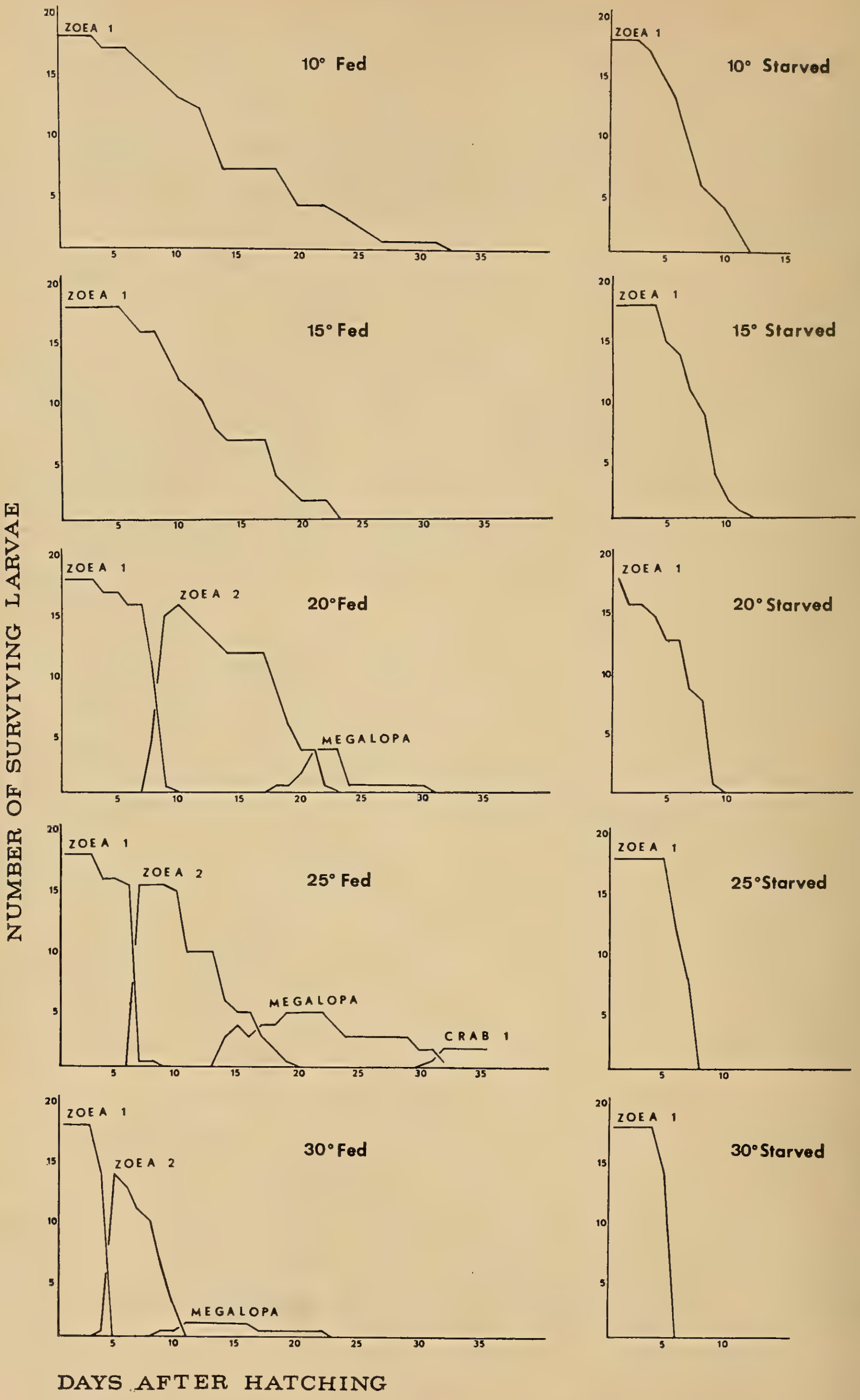
NUMBER OF SURVIVING LARVAE
DAYS AFTER HATCHING
10° Fed
10° Starved
15° Fed
15° Starved
20° Fed
20° Starved
25° Fed
25° Starved
30° Fed
30° Starved
ZOEA 1
ZOEA 2
MEGALOPA
CRAB 1
20
15
10
5
5
10
15
20
25
30
35

FIGURE 1.

20 m. offshore of Hurricane Harbor, Key Biscayne, Florida. The crabs were isolated in non-flowing sea water in 19-cm. diameter plastic bowls until hatching occurred. Zoeae were placed, one each, in compartmented plastic tackle boxes. Each compartment held about 40 cc. of unfiltered sea water (33–35‰ salinity). Two series of boxes, one with zoeae fed with *Artemia salina* nauplii, and one with starved zoeae, were maintained at 10°, 15°, 20°, 25°, and 30° C. Temperature variation was not more than ± 1.5° C. Larvae were fed and received a change of water every other day. Illumination was not controlled. A record was kept for all molts and deaths for each larva, with exuviae and dead larvae preserved in 70% ethanol. Larvae obtained from another female were held at 25° C. (± 2° C.) and several zoeae were sacrificed every day, and also preserved in 70% ethanol. Appendages were dissected in 40% lactic acid and mounted in Turtox CMC-S. Illustrations were made with a camera lucida attached to a Wild M-20 binocular compound microscope, from slides of individual appendages. Drawings were checked both for accuracy and individual larval variation against appendages dissected from exuviae and sacrificed animals. Measurements were made with a LaFayette objective micrometer. Carapace length was measured from the anterior margin of the zoeal eye to the insertion of the posterior spines on the zoeal carapace, and from the edge of the megalopal frontal region to the posterior margin of the carapace for carapace length, and across the widest part of the carapace for carapace width. The carapace measurements provided are the arithmetic average of 10 specimens measured in each larval stage. One spent female (UMML 32:3581) plus hatched specimens of first and second zoeae and megalopae (UMML 32:3582) were placed in the Museum of the Institute of Marine Sciences.

RESULTS

Polyonyx gibbesi passes through a pre-zoeal stage, two zoeal stages and one megalopal stage. As noted for other porcellanids (*e.g.* Knight, 1966) there is an increase in size of the third maxillipeds, gills and pleopods during the zoeal stages. Some authors (*e.g.* Boschi *et al.*, 1967) consider this increase a substage, though no molt is seen. Others (*e.g.* Boyd and Johnson, 1963) refer to molting that produces additional stages but little or no alteration of form as substages. The term substage needs re-definition. I never observed exuviae other than the two zoeal molts, and I do not use the term substage.

Temperature noticeably affects larval development, altering the duration of each stage or preventing the development to subsequent stages. Figure 1 depicts the length of time the larvae, both fed and starved, spent in each stage at various temperatures. No starved zoeae developed beyond the first stage. Fed zoeae at 10° and 15° C. remained in Stage I, living at 10° C. for a maximum of 32 days, and at 15° C. for a maximum of 22 days, before dying. Animals reared at 20°, 25°, and 30° C. reached the megalopal stage in a minimum of 18, 14 and eight days, respectively. Crab stages were obtained only at 25° C. While the

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FIGURE 1. *Polyonyx gibbesi:* Duration of survival in each stage of larval development, fed and starved, at various temperatures. Horizontal scale represents the number of days after hatching. The vertical scale represents the number of surviving larvae. Salinity range = 33–35‰.

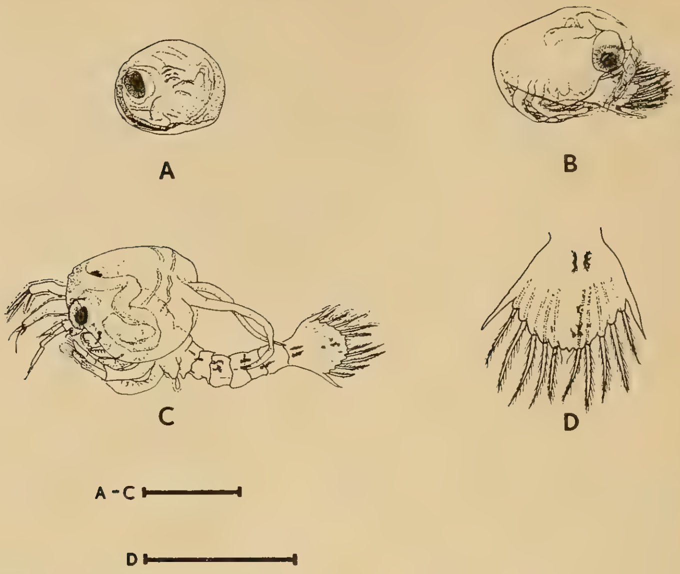

FIGURE 2. *Polyonyx gibbesi:* pre-zoea. A. Larva in egg just before hatching. B. Larva in process of hatching. C. Newly hatched pre-zoea. Remnants of pre-zoeal cuticle surround appendages. Rostral spine and setae on appendages not yet emerged. D. Pre-zoeal telson. Two hairs on central prominence of telson and other fine setae not yet present. Scales equal 0.5 mm.

shortest duration of development occurred at 30° C., no crab stages were obtained. Mortality at this temperature was very high and only three zoeae molted to megalopa and none of these survived longer than 11 days. Thus, 25° C. allowed the best development in the temperature series. At this temperature the first and second zoeal stages lasted six to seven days and the megalopal stage usually lasted 12 to 14 days.

DESCRIPTION OF THE LARVAE

Pre-zoea

The hatching sequence of the entire egg mass lasted about two hours (see Fig. 2, A, B). The pre-zoeal stage also lasts about two hours. The long rostral spine, in the pre-zoea as yet undeveloped, is partially bent outside and under the carapace and partially invaginated into the carapace above the midgut region (Fig. 2, C). The embryonic cuticle in the specimens examined was almost completely fragmented. Setae on the appendages and telson, only partly extruded at the beginning of the stage, become completely extruded toward the end of the stage. The setae on the carapace over the eyes and setae on the central prominence of the telson (Fig. 2, D) are not present. The pre-zoeae swim by rapid abdominal flexion.

In two instances pre-zoeae swam from *Chaetopterus* tubes maintained in the laboratory. If the larvae did not hatch as prezoeae they might encounter difficulty in escaping through the narrow neck of the worm tube.

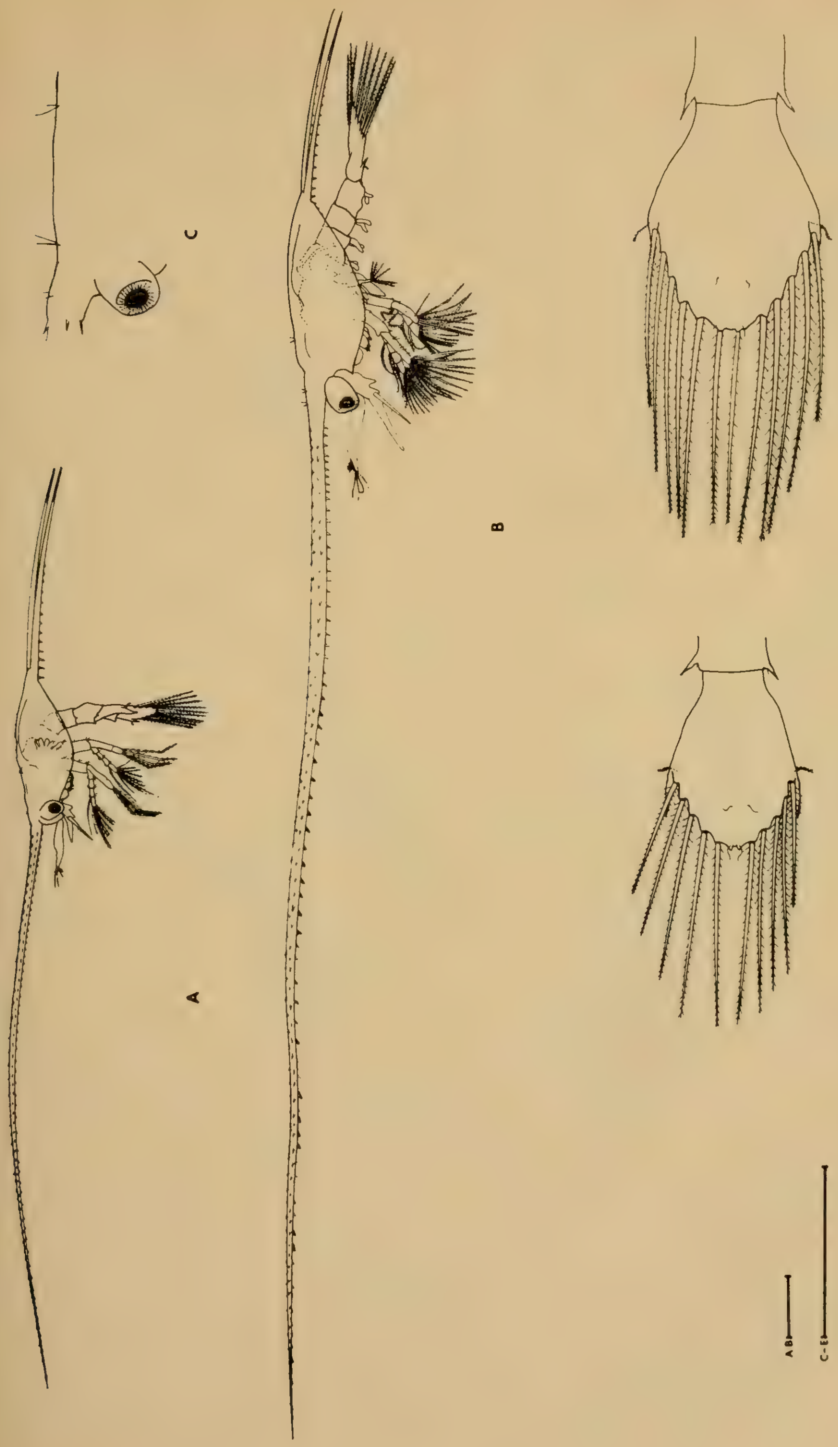

FIGURE 3. *Polyonyx gibbesi*: first and second zoeal stages. A. First zoea. B. Second zoea. C. Caparapace of first zoea showing dorsal setae. D. Telson of first zoea. E. Telson of second zoea. Scales equal 0.5 mm.

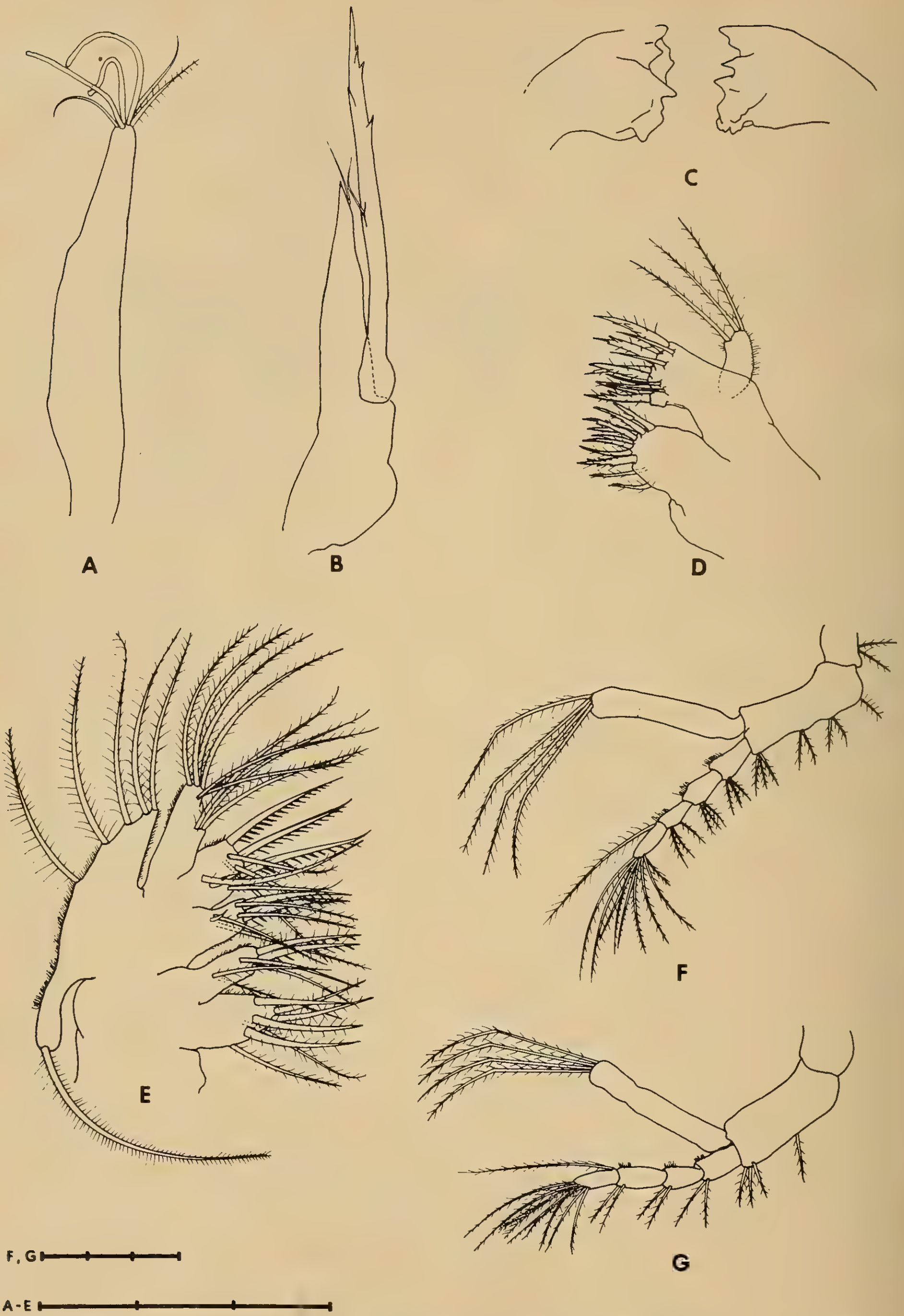

FIGURE 4. *Polyonyx gibbesi;* first zoeal appendages. A. Antennule. B. Antenna. C. Mandibles. D. Maxillule. E. Maxilla. F. Maxilliped 1. G. Maxilliped 2. Scales equal 0.3 mm.

First Zoea

Carapace length: 1.2 mm.

Number of specimens examined: 20

Carapace (Fig. 3, A). Typically porcellanid, produced anteriorly into an extremely long rostral spine up to seven times the length of carapace proper; posteriorly into two straight or divergent posterior carapace spines 1.4 to 1.8 times as long as carapace. Armature of spines as illustrated. Curvature of rostral and posterior carapace spines variable, depending in part on frequency of collision by new zoeae with other objects.

Dorsal surface of carapace with three pairs of fine setae. Placement illustrated in Figure 3, C.

Antennule: (Fig. 4, A). A simple slightly flabelliform rod with two long and one short aesthetasc and two or three setae of variable length, one with fine setules.

Antennae: (Fig. 4, B). Endopodite, fused to protopodite, has a thin subterminal seta. Exopodite a thin spine almost twice as long as endopodite; about six small spinelets distally plus a thin seta halfway down its length.

Mandibles: (Fig. 4, C). Asymmetrical dentate processes without palp.

Maxillule: (Fig. 4, D). Endopodite unsegmented, 3 setae. Coxal and basal endites each with 10 processes as shown.

Maxilla: (Fig. 4, E). Endopodite unsegmented, 4 terminal, two subterminal and three medial setae. Coxal endite with seven processes on proximal lobe, six on distal lobe. Basal endite with seven processes on proximal lobe, nine on distal lobe. Scaphognathite with five setae laterally and one long apical plumose seta. Placement of all setae as illustrated.

Maxilliped 1: (Fig. 4, F). Four and ten setae on terminal segments of exopodite and endopodite, respectively. Basipodite setation progressing distally is 1, 2–3, 2, 3. Small tufts of hair dorsally on endopodite segments one to three.

Maxilliped 2: (Fig. 4, G). Setation on terminal segments similar to maxilliped 1. Basidopodite setation 1–2, 3 progressing distally. Tufts of hair dorsally on segments one to three of endopodite as in maxilliped 1.

Maxilliped 3: (Fig. 3, A). A small bifid lobe, with one or two setae.

Pereiopods: Five buds visible in most specimens; extremely small and distorted in early stage. Both buds and maxilliped 3 increase in size as zoea progresses to Stage II.

Abdomen: (Fig. 3, A). Five somites, each with lateral spine of increasing length on somites nearer the telson. Somites three and four may each have a fine hair dorso-laterally; somite five has two fine setae just above lateral spine on each side.

Pleopods: Absent. Primordia visible in some zoeae from the plankton.

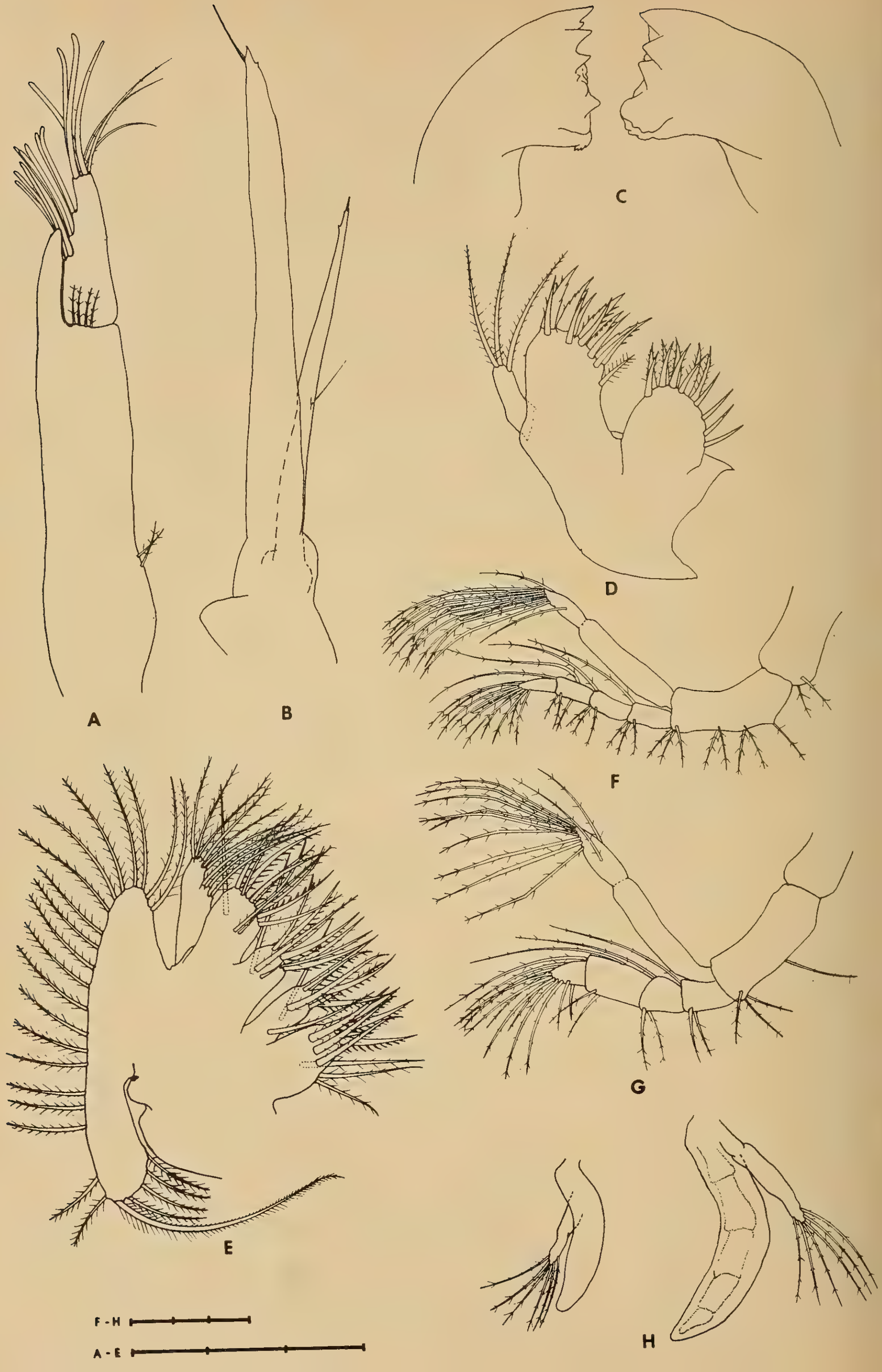
A
B
C
D
E
F
G
H
F-H
A-E

FIGURE 5.

Telson: (Fig. 3, D). Distinctive characters are minutely serrated lateral spines, the thin seta next to each telson spine, two fine hairs on central prominence, minute serrations terminally on articulated plumose setae, and two fine hairs medially on dorsal surface of telson proper. Anal spine present.

Color: Zoea transparent with red-orange chromatophores as follows: dorsally and ventrally surrounding the gut throughout abdomen; dorsally between lateral spines on telson; dorsally on tip of telson; interiorly around mouthparts. Rostral spine diffusely red-orange at tip and intermittently so throughout its length; posterior carapace spines diffusely red-orange only at tips.

Second zoea

Carapace length: 1.7 mm.

Number of specimens examined: 15

Carapace: (Fig. 3, B). Three pairs of dorsal setae persist. Rostral spine about six times carapace length; posterior spines of carapace up to 1.6 times carapace length. Spination on ventral margin of posterior spines may extend onto carapace in some zoeae.

Antennule: (Fig. 5, A). Biramous; exopodite with three or four long aesthetascs and four terminal setae, one seta plumose. Subterminal aesthetascs arranged in three groups as 2, 3, 3 progressing proximally. Endopodite slightly less than half the length of exopodite. At junction of exopodite and endopodite are four small setate; two additional setae on basal medial projection of protopodite.

Antenna: (Fig. 5, B). Similar to Stage I. Exopodite now about $\frac{2}{3}$ as long as endopodite; distal spination almost absent.

Mandibles: (Fig. 5, C). Larger, with three or four teeth and smaller dentate processes. No evidence of palp though Faxon (1879) reported one present and illustrated it as rudiment.

Maxillule: (Fig. 5, D). Endopodite setation same as in Stage I. Coxal and basal endites with 12 processes each, as illustrated. One late stage zoea with one more seta on lateral margin of coxal endite making 13 processes.

Maxilla: (Fig. 5, E). Endopodite setation same as Stage I. Coxal endite with 17–18 setae, 10 on proximal lobe, seven or eight on distal lobe. Basal endite with nine setae on proximal lobe, 11 on distal lobe. Scaphognathite retains apical plumose seta, and now has about 24 setae on margins.

Maxilliped 1, 2: (Fig. 5, F-G). Terminal segments of exopodite and endopodite with about 12 setae. Dorsal tuft of hairs on endopodite segments replaced by single long seta on each segment. Third segment of endopodite of Maxilliped 2 much swollen and nearly twice as long as other segments. Other setation similar to Stage I.

FIGURE 5. *Polyonyx gibbesi;* second zoeal appendages. A. Antennule. B. Antenna. C. Mandibles. D. Maxillule. E. Maxilla. F. Maxilliped 1. G. Maxilliped 2. H. Maxilliped 3, early (l.) and late (r.) stage. Scales equal 0.3 mm.

Maxilliped 3: (Fig. 5, H). Exopodite indistinctly segmented with six terminal setae. Endopodite, the same length or slightly longer than exopodite after molting, increases in length throughout second stage. It measures three times the length of exopodite just before molt to megalopa.

Pereiopods: One and five indistinctly chelate. Gills present. Pereiopods and gills enlarge noticeably throughout duration of Stage II. Toward end of Stage II the almost completely formed pereiopods are tucked under posterior portion of carapace.

Pleopods: (Fig. 3, B). Buds present, of decreasing length on abdominal segments two through five. Buds increase in size as stage progresses.

Telson: (Fig. 3, E). Now with two long articulated plumose setae on central prominence making 8 + 8 processes. Fine hairs below prominence and those adjacent to lateral spines are retained. Setation on dorsal surface unchanged from Stage I.

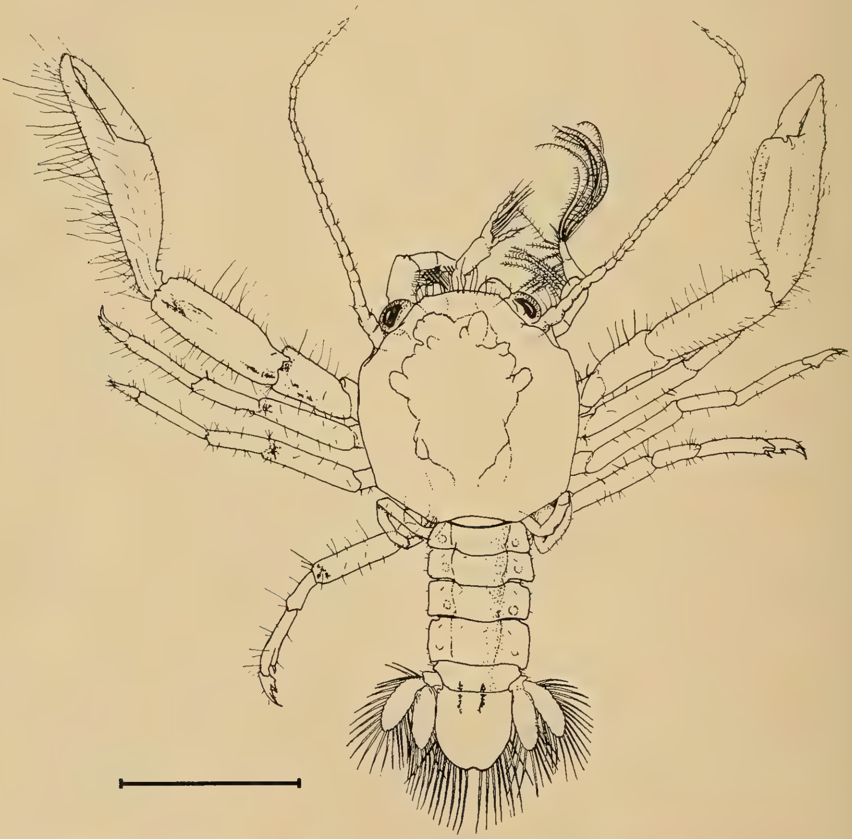

FIGURE 6. *Polyonyx gibbesi;* megalopa. Right antennule and left maxilliped 3 removed for clarity. Scale line equals 1 mm.

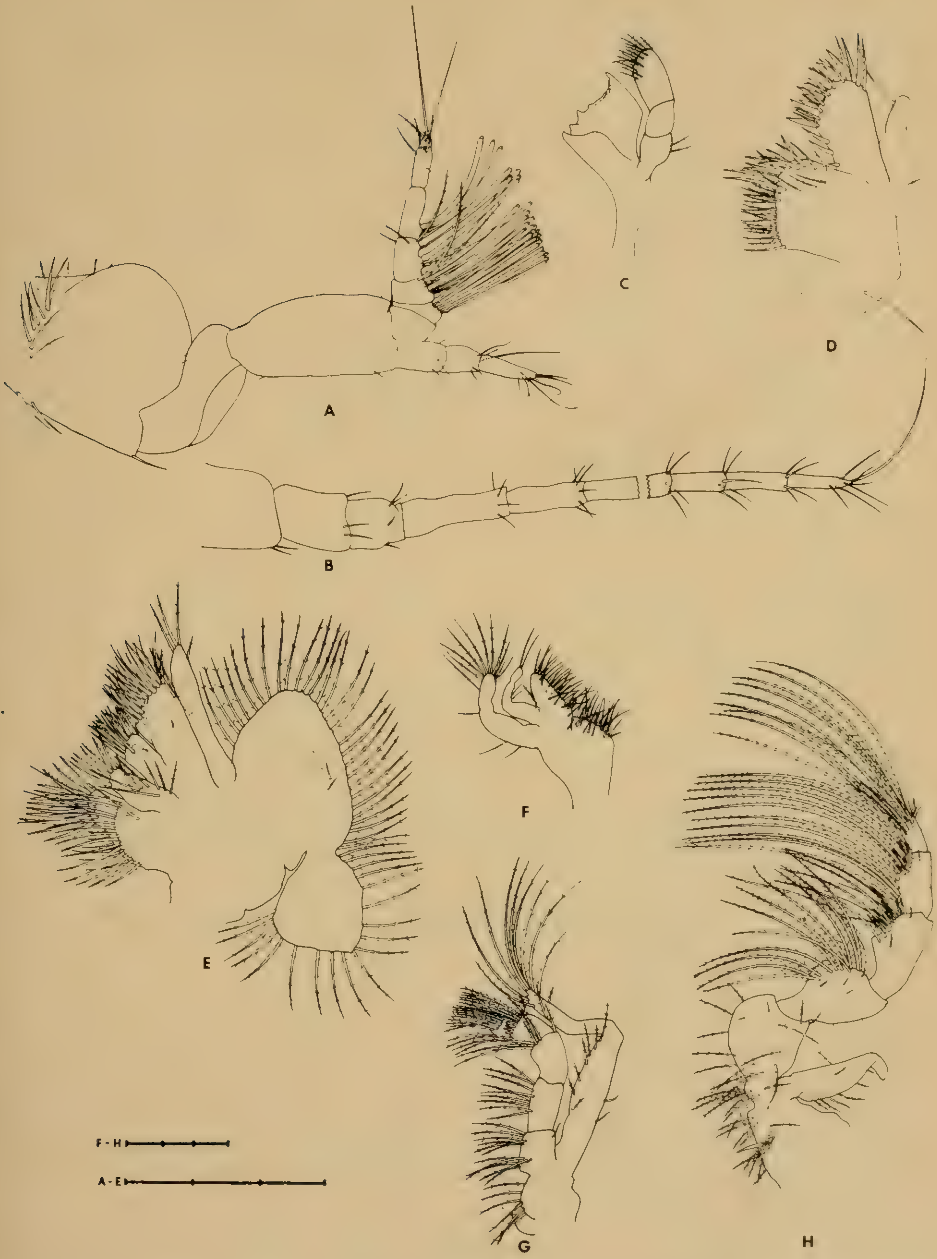

FIGURE 7. *Polyonyx gibbesi;* megalopal appendages. A. Antennule (less tips of aesthetascs). B. Antenna (in part). C. Mandible. D. Maxillue. E. Maxilla. F. Maxilliped 1. G. Maxilliped 2. H. Maxilliped 3. Scales equal 0.3 mm.

Color: Chromatophore distribution and color similar to Stage I. As zoea approaches molt to megalopa, rostral spine becomes completely orange. Posterior spines of carapace colored orange but to lesser extent.

Megalopa

The megalopa (Fig. 6) resembles the adult crab closely, enough so that Faxon (1879, 1882) considered it the "first stage of the crab." And, though he acknowledged the presence of biramous pleopods on the abdomen, to substantiate his belief he also cited a lack of persistent zoeal characters expected to appear in a megalopa stage. Because of this, at least one author (*e.g.,* Williams, 1965: 114) has quoted Faxon's error without further evaluation.

Chelae are well developed and fringed with setae on their outer margin. The juvenile and adult crab is always broader than long whereas only in the megalopa are the animals longer than broad. Carapace width-to-length measurements ranged from 1.2 mm. × 1.2 mm. to 1.4 mm. × 1.4 mm. First crab measurements were 1.8 mm. wide by 1.6 mm. long.

Carapace: (Fig. 6). Rounded or somewhat quadrate. Frontal region little produced; bears numerous setae. Eyes relatively large compared to first crab stage.

Antennule: (Fig. 7, A). Biramous, with three-segmented peduncle; basal segment enlarged. Lower ramous three-segmented; upper ramous has seven segments with aesthetascs on segments two through five in the following sequence of rows and numbers: one row (10), two rows (10, 3, +2 setae), two rows (3, 2, +1 seta), one row (3). Other setation on both rami as illustrated.

Antenna: (Fig. 7, B). Three-segmented peduncle plus 25 short segments, each bearing several short setae. Terminal segment usually with a long seta.

Mandibles: (Fig. 7, C). Three-segmented palp present, first segment has two setae on distal edge; distal segment with approximately 15–20 strong setae and spines.

Maxillule: (Fig. 7, D). Endopodite two-segmented, with setae as shown. Basal and coxal endites have approximately 29 and 36 processes, respectively, placed as shown.

Maxilla: (Fig. 7, E). Endopodite unsegmented; three or four terminal, two or rarely three subterminal setae. Proximal lobe of coxal endite with at least 10 and up to 13 terminal processes, three subterminal processes and about 20 setae in a ring around middle of lobe. Distal lobe with six or seven terminal and three subterminal processes; seven setae progress down its side. Proximal lobe of basal endite with about 15 processes; distal lobe has about 30. Scaphognathite has 48 or more plumose setae around edge.

Maxilliped 1: (Fig. 7, F). Setation fragile and variable; expodite with two to ten setae; endopodite with four to six setae; protopodite with 50 or more setae on coxal and basal lobes.

Maxilliped 2: (Fig. 7, G). Exopodite elongate, about 20 setae on its two segments. Setation on four-segmented endopodite progressing distally is 9, 8, 18–20,

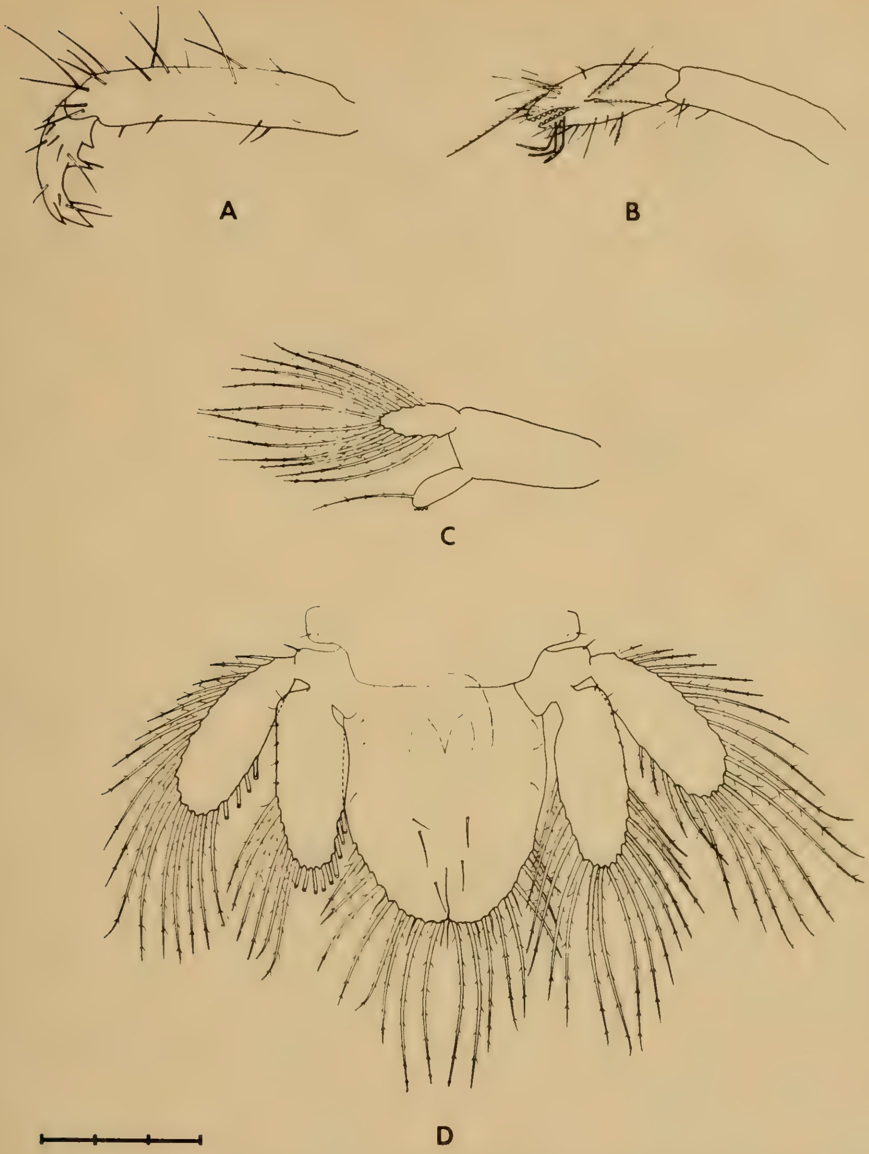

FIGURE 8. *Polyonyx gibbesi;* locomotory appendages of megalopa. A. Pereiopod 2. B. Pereiopod 5. C. Pleopod. D. Tail-Fan. Scale equals 0.3 mm.

about 18. Basipodite and coxopodite with about six and 12 setae, respectively. Placement as illustrated.

Maxilliped 3: (Fig. 7, H). Numerous setae on inner margin of basal lobes; about 14 setae and small spine on coxal lobe, as shown. Processes on five-segmented endopodite as follows: ischium, about 14; merus, about 16; carpus, about 18; propodus, about 23; dactylus, about 15. Ischium, merus and carpus have thin, platelike extensions. Exopodite has six to eight setae, placed as illustrated.

Pereiopods: (Fig. 6; 8 A, B). Chelipeds large, flattened, subequal, somewhat distorted, covered with setae. Walking legs setose, with two small blunt spines on distal edge of propodus (spines may be poorly developed); dactyl with three or four accessory spines. Pereiopod five chelate, gape appearing dentate in some, approximately 25 setae and three pectinate scythe-like hooks near gape.

Pleopods: (Fig. 8, C). Biramous, of decreasing size on abdominal somites nearer the telson. Exopodites with usually 12 setae; endopodites with one subterminal seta and an appendix interna.

Telson: (Fig. 8, D). 8 + 8 plumose setae plus additional spines and setae as

TABLE I

Comparison of zoeal appendages in two species of Polyonyx. Data for P. quadriungulatus from Knight (1966) and for P. gibbesi from the present work.

	Zoea I		Zoea II	
Appendage	*P. gibbesi*	*P. quadriungulatus*	*P. gibbesi*	*P. quadriungulatus*
ANTENNULE	Simple rod	Simple rod	Biramous	Biramous
Exopodite	3 aesthetascs; 2–3 setae	3 aesthetascs; 3 setae	11 aesthetascs; 4 setae	11 aesthetascs; 4 setae
Endopodite			1/2 length of exopodite	1/2 length of exopodite
ANTENNA	Biramous	Biramous	Similar to Stage I	Similar to Stage I
Exopodite	2 × length of endopodite; spinous	3 × length of endopodite; spinous	2/3 length of endopodite Spination reduced	About equal to endopodite Spination as in Stage
Endopodite	1 subterminal seta	1 subterminal seta	As in Stage I	As in Stage I
MANDIBLE	Asymmetrical simple processes	Asymmetrical simple processes	3 large teeth No palp	2 teeth figured No palp
MAXILLULE				
Endopodite	3 apical setae	3 apical setae	3 setae	3 setae
Coxal endite	4 spines; 6 setae	4 spines; 6 setae	5 spines; 7 setae	5 spines; 7 setae
Basal endite	6 spines; 4 setae	6 spines; 4 setae	7 spines; 5 setae	7 spines; 5 setae
MAXILLA				
Endopodite	9 setae	9 setae	9 setae	9 setae
Coxal endite	13 processes	13 processes	17–18 processes	17 processes
Basal endite	16 processes	16 processes	20–21 processes	20 processes
Scaphognathite	5 setae; 1 spine	5 setae; 1 spine	24 (+1 apical) setae	21–26 (+1 apical) setae
MAXILLIPED 1				
Coxopodite	2 setae	2 setae	2 setae	2 setae
Basipodite	1,2—3,2,3, setae	1,2,2,3 setae	1,2,2,3 setae	1,2,2,3 setae
Endopodite	3,3,3,10 setae	3,3,3,8 setae	4,4,4,12 setae	4,4,4,9 setae
Exopodite	4 natatory setae	4 natatory setae	12 setae	11–12 setae
MAXILLIPED 2				
Coxopodite	1 seta or naked	Naked	1 seta or naked	Naked
Basipodite	1–2,3 setae	1,2 setae	1,3 setae	1,2 setae
Endopodite	2,2,2,10 setae	2,2,2,8 setae	3,3,3,12 setae	3,3,3,9 setae
Exopodite	4 natatory setae	4 natatory setae	12 setae	11–14 setae
MAXILLIPED 3	Small bifid lobe	Rudimentary	Now functional	Now functional
Endopodite			Increases in length	Increases in length
Exopodite			2 indistinct segments; 6 setae	2 indistinct segments; 6 setae
PEREIOPODS	Present as buds	Present as buds	Developing Chelation seen	Developing Chelation seen
ABDOMEN	5 somites with lateral spines	5 somites with lateral spines	Pleopod buds present Increase in size seen	Pleopod buds present Increase in size seen
TELSON	7 + 7 processes; 2 hairs on central prominence	7 + 7 processes; 2 hairs on central prominence	8 + 8 processes, hairs on prominence retained	8 + processes, hairs on prominence retained

shown. Uropods biramous, exopodites with 18–24, endopodites 12–16, setae around distal margins.

DISCUSSION

At present the complete larval development is known for only two of the 23 described species of *Polyonyx,* one from the eastern Pacific and one from the western Atlantic. A comparison between the eastern Pacific *Polyonyx quadriungulatus* and *P. gibbesi* shows that the zoeal stages are almost exactly similar in number and placement of setae (see Table I). *P. gibbesi* differs most notably in having more setae on the terminal segments of the endopodites of the maxillipeds in both zoeal stages (10 and 12) than *P. quadriungulatus* (8 and 9). Only detailed examination of each appendage reveals further differences between the zoeae of the two species (*e.g.,* different number of setae and spines on the second maxillae).

More easily observed is the relative length of the antennal exopodite to the endopodite in the two species (see Table I). Knight (1966) considered this a good character for distinguishing *P. quadriungulatus* from *Porcellana* and *Pisidia,* the two other members of Lebour's (1943) triad relationship. It is also possible on this basis to separate *P. gibbesi* from *P. quadriungulatus.* In the latter the antennal exopodite is three times the length of the endopodite in Stage I zoea, becoming about equal to the endopodite in Stage II. In *P. gibbesi* the antennal exopodite is only twice as long as the endopodite in Stage I and becomes $\frac{2}{3}$ the length of the endopodite in Stage II. Thus, the exopodite is always shorter in the zoeal stages of *P. gibbesi* than in *P. quadriungulatus.*

A third character which distinguishes *P. gibbesi* from *P. quadriungulatus* is seen in the dorsal setation of the zoeal carapace. The eastern Pacific form has but two setae in both zoeal stages while *P. gibbesi* has three pairs in both stages. Setae on the dorsal surface of the telson, often difficult to observe, may be an additional feature to separate larvae of the two species.

Differences in the megalopae of the two species are less distinct. The mouthparts are quite similar in both form, and number and placement of setae. A comparison of the megalopae in Table II shows that detailed examination is again necessary to separate the two forms. In general, however, *P. gibbesi* has more setae on the mouthparts than *P. quadriungulatus.* It also lacks both the two small spines at the bases of long setae and the articulated spines on the posterior distal margin of the propodus which *P. quadriungulatus* possesses.

The adult morphology of the two species is quite similar. Haig (1960, p. 239) stated that "Aside from *Polyonyx nitidus* Lockington, *P. quadriungulatus* is most closely related to . . . *P. gibbesi.*" Further, both *P. gibbesi* and the two California species just mentioned belong to Johnson's (1958) "*P. sinensis* group" (Haig, 1960, p. 238). This group is a complex of species from the Indo-Pacific (and now including California to Panama, the eastern U. S., and the west African coast, Haig, *in Litt.*) which show similar morphology, plus "a pronounced tendency toward commensalism" (Johnson, 1958, p. 97). *P. gibbesi* is considered an obligate commensal with the polychaete worm *Chaetopterus variopedatus* (Gray, 1961), the megalopa establishing the initial relationship with the worm (Gore, unpublished data). Both California species have been found commensal with *Chaetopterus* though they may not be obligate commensals.

TABLE II

Comparison of megalopa appendages in two species of Polyonyx. Data for P. quadriungulatus from Knight (1966) and for P. gibbesi from the present work.

Appendage	*P. gibbesi*	*P. quadriungulatus*
ANTENNULE	Biramous	Biramous
	3 segmented peduncle, basal segment enlarged	3 segmented peduncle, basal segment enlarged
Ventral ramus	3 segments	3 segments
Dorsal ramus	7 segments; 10, 10 + 3, 3 + 2, 3 aesthetascs in tiers	7 segments; 10, 10 + 3, 3 + 2, 3 aesthetascs in tiers
ANTENNA	About 25 segments	About 30 segments
	3 segmented peduncle	3 segmented peduncle
	Small lobe on first peduncular segment	Small cylindrical branch on first peduncular segment
MANDIBLE	3 segmented palp	3 segmented palp
Distal segment	About 20 setae	15–17 setae
MAXILLULE		
Endopodite	2 segments; 1–2 setae	2 segments; 1–2 setae
Basal endite	14 spines, 15 setae	10 spines, 21 setae*
Coxal endite	10 spines, 26 setae	About 44 processes*
MAXILLA		
Endopodite	Unsegmented, 7 setae	Unsegmented, 8 setae
Basal endite	45 processes	41 processes*
Coxal endite	49 processes	43 processes*
Scaphognathite	About 47 setae	48–58 setae
MAXILLIPED 1		
Protopodite	50 or more setae	About 50 setae
Endopodite	6–7 setae	7 setae
Exopodite	10 or 11 setae	13–20 setae
MAXILLIPED 2		
Protopodite	18 setae	10 setae*
Endopodite	4 segments; 9, 8, 18–20, 18 setae (in tufts on segs. 3–4)	4 segments; —, 8, in tufts, in tufts
Exopodite	2 segments; 11 terminal, 9 marginal setae	2 segments; 11 terminal, 7 marginal setae
MAXILLIPED 3		
Protopodite	Serrated spine, numerous setae	Serrated tooth, numerous setae
Endopodite	5 segments; about 14, 17, 21, 27, 17 processes	5 segments; 15, 14, 20, 12, ? processes*
Exopodite	6 terminal, 6 marginal setae	6 terminal, 6 marginal* setae
PEREIOPODS 2–4	Dactyls with 3–4 hooks; propodus may have 2 small spines distally	Dactyls with 4 fixed hooks; propodus with 2 articulated spines
PEREIOPOD 5	Chelate; 26 setae + 3 scythe-like hooks	Chelate; 1 spine, 2–3 scythe-like hooks + setae
PLEOPODS 1–4		
Endopodites	1 subterminal seta; 4-5 terminal hooks	1 subterminal seta; 4–5 terminal hooks
Expodites	Usually 12 setae	12, 12–13, 13, 13 setae
UROPODS		
Endopodite	10–12 setae	10–13 setae
Exopodite	18–22 setae	17–22 setae
TELSON	8 + 8 plumose setae, + spines	8 + 8 plumose setae, + spines

* No specific description given. Data interpolated from illustrations.

The similarity in adult and larval morphology, the geographical isolation between Pacific and Atlantic forms, plus the similarity in commensal habitat indicates that *P. gibbesi* and *P. quadriungulatus* are geminate species. Thus they are one more species pair of the many that are known to exist between Caribbean-Atlantic and Pacific coast forms (*e.g., Minyocerus kirki* and *angustus, Porcellana cancrisocialis* and *sayana,* etc., see Haig, 1960).

Lebour (1943) thought that Faxon's *Polyonyx macrocheles* would fit into the *Porcellana-* (and *Pisidia*) *-Polyonyx* complex, distinguished chiefly by the placement of the fifth plumose setae of the telson in the first and second zoeal stages. Knight (1966) showed that *Polyonyx quadriungulatus* adhered to Lebour's scheme and the present work confirms Lebour's suggestion for *P. gibbesi* (formerly *P. macrocheles*). The differences in antennal proportions between *Porcellana-Pisidia* and *Polyonyx quadriungulatus,* as noted by Knight (see above), apply also to *P. gibbesi.* Thus, the zoeal features such as spined antennal exopodite and its length relative to both the antennal endopodite and to the antennule allow *P. gibbesi* to be separated from *P. quadriungulatus* in both zoeal stages, as already discussed, and from the known species of *Porcellana* and *Pisidia* in the first zoeal stage. The scheme breaks down in the second zoeal stage since, in *P. gibbesi,* the antennal exopodite is ⅔ as long as the endopodite while the endopodite is about as long as the antennule proper. *Polyonyx gibbesi* thus shows antennal characters (in length) similar to those shown by known species of *Porcellana* and *Pisidia* in the second zoeal stage.

The previously mentioned carapace setation may, however, allow complete separation of *Polyonyx gibbesi* in both zoeal stages from *Porcellana-Pisidia* zoeae. If it is consistent in other members of the genus *Polyonyx* then, together with the features mentioned above, it would make *Polyonyx* larvae immediately distinguishable from most other porcellanid larvae. The value of this last character must await further studies on the larvae of other genera since some western Atlantic species of *Pachycheles, Petrolisthes, Porcellana* and *Minyocerus* also have dorsal carapacial setation (Gore, unpublished data). Studies are presently being carried out on the larvae of other genera of Porcellanidae from the south Florida and Caribbean area. Each of the genera mentioned has good distinguishing features in the zoeae which, in conjunction with Lebour's characters regarding the telson, may allow them to be separated from one another (Gore, unpublished data). As larvae of these genera become better known it will be possible to construct a key for their identification and to clarify the relationships between them.

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Summary

1. The larval development of the porcellanid crab, *Polyonyx gibbesi*, a commensal with the polychaete worm *Chaetopterus variopedatus*, is described and illustrated. Two series of larvae were hatched and maintained in the laboratory, one fed with *Artemia* nauplii and the other starved. Members of each series were held at 10°, 15°, 20°, 25° and 30° C. At 25° C. the fed larvae hatched as prezoeae and molted through two additional zoeal stages to the megalopa. Duration of the pre-zoeal stage is about two hours, each of the zoeal stages usually lasts six to seven days and the megalopa lasts 12–14 days before molting to first crab. No crab stages were obtained above or below 25° C. and no megalopae were obtained below 20° C. Starved larvae died before attaining Stage II.

2. Comparison of the larvae of *Polyonyx gibbesi* with those of *Polyonyx quadriungulatus*, an eastern Pacific species, shows the zoeae and megalopae to be almost identical both in appendages and in form, numbers, and placement of setae. Similarity of morphology and habitat plus geographical isolation indicate that *P. gibbesi* and *P. quadriungulatus* are geminate species.

3. Larvae of *Polyonyx gibbesi* possess certain features which allow them to be recognized in the plankton as well as distinguished from known larvae of genera of other western Atlantic porcellanid crabs.

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COMPENSATION OF LIVER PROTEIN SYNTHESIS IN TEMPERATURE-ACCLIMATED TOADFISH, OPSANUS TAU

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In the process of temperature acclimation poikilotherms adjust their metabolic rates in order to maintain physiological activity at a more nearly constant level over a range of environmental temperatures. Thus, they attain a measure of independence of temperature (Prosser and Brown, 1961). A review of the earlier literature dealing with this phenomenon and discussion of its significance in ecology and evolution has been given by Bullock (1955). More recently, the subject has been reviewed in several symposia (Prosser, 1958; 1967; Troshin, 1967). Most studies have dealt with temperature acclimation in terms of physiological (heart rate, oxygen consumption) or behavioral parameters; it is only in the last few years that methods have become available to attempt an approach to the molecular basis of the phenomenon.

Several recent studies have presented evidence for changes in level of enzymes in response to acclimation. Freed (1965) demonstrated an increase in cytochrome oxidase activity in goldfish muscle as measured at 15° after the fish had been acclimated to 5°; decreased activity resulted from warm acclimation (25°). Similarly, increased 6-phosphogluconic dehydrogenase activity is found in gills of cold-acclimated carp (Ekberg, 1962). Hochachka and Hayes (1962) showed greater utilization of the pentose shunt in cold-acclimated trout. Changes in the lactic dehydrogenase isozyme system have been found to accompany thermal compensations in goldfish (Hochachka, 1965). Because of the probability that these activity increases are due to increased levels of enzyme protein, a study was begun on the effect of low temperature acclimation on protein synthetic capacity in fish. The toadfish was chosen as the experimental animal because of its ready adaptability to laboratory conditions, and its wide range of natural habitat (Bigelow and Schroeder, 1953; Henshall, 1891), suggesting a high degree of temperature adaptability. Studies were concentrated on the liver where (in the case of rat) a good deal has been learned about the mechanism of protein synthesis. Of equal importance to the question of the mechanism of temperature acclimation is the possibility that the phenomenon can be used to elucidate sites of regulation in the protein synthetic system *per se*. A preliminary report of this work has appeared (Haschemeyer, 1967).

Materials and Methods

Animals

Adult toadfish, 200–300 g., were obtained from the Supply Department at the Marine Biological Laboratory, Woods Hole, and included both sexes. In the first series of experiments the fish were kept in running sea water aquaria at the Marine Biological Laboratory. With the available refrigeration system it was possible to keep groups of up to six fish at a temperature of 10° ± 1° in running sea water during the period of low temperature acclimation. Control fish were kept at 20–22°, the laboratory temperature of the sea water supply at Woods Hole at that time. In the second series of experiments the fish were maintained in aerated static aquaria prepared with a synthetic sea salt mixture (Rila Products, Teaneck, N. J.). For low temperature acclimation the fish were kept in a 25-gal. Instant Ocean aquarium (Aquarium Systems, Inc., Wickliffe, Ohio) with temperature control at 10° ± 0.5°. Control fish were kept at room temperature, 22–24°. The toadfish were fed small *Fundulus heteroclitus* to appetite.

Measurement of in vivo protein synthesis

For determination of incorporation of C^{14} amino acids into liver protein, a rapid arterial injection route was used, as described by Cooperstein and Lazarow (1964). In cases where the incubation temperature for the measurement differed from the acclimation temperature, the fish was transferred to the new temperature $\frac{1}{2}$–1 hr. before injection. Each fish was wrapped in cheesecloth with its tail brought around to the right of the body, and a hole was cut in the cloth over the left gill. The skin on either side of the operculum was clipped in order to expose the gill arches and 0.2 ml. of a solution containing 10 μc. of C^{14} amino acids was injected through a 25-gauge, $\frac{5}{8}''$ needle into the branchial artery of the fourth gill arch. The isotope solution was prepared from a mixture of 15 purified C^{14}-L-amino acids (New England Nuclear Corp.) of high specific activity (1 mc./mg.); the commercial solution in 0.1 N HCl was neutralized, buffered with 0.5 *M* Tris, pH 7.4, and brought to 1% NaCl. After injection the gill flap was held closed for about 5 seconds to aid clotting at the needle hole, and the fish was returned to a pail of sea water for the incubation period. The entire procedure took about 2 minutes; the fish started swimming as soon as returned to water and showed no ill effects. After time intervals of 5 min. to 30 min. the fish was stunned by a blow to the head, and the liver quickly excised and transferred to cold Medium A (0.25 *M* sucrose, 0.05 *M* Tris, pH 7.4, 0.025 *M* KCl, 0.01 *M* $MgCl_2$). In some cases to determine free radioactivity in the blood as a measure of the effectiveness of the injection, approximately 0.7 ml. of blood was collected by syringe from the heart cavity and added to 0.1 ml. of 0.1 *M* ethylenediamine tetraacetate, K^+, pH 8.0.

Each liver was accurately wet-weighed, minced and homogenized in 3 volumes cold Medium A in a Sorvall Omni-mixer at half of full speed. For the second series of experiments in which biochemical analyses were made, the livers were homogenized in 3 volumes of ice-cold distilled water. The homogenates were analyzed for C^{14} incorporation into protein using a filter-paper disc method, based on the method of Bollum (1959). For each homogenate 100-μl. aliquots were pipetted onto several discs (Whatman 3MM filter paper, 2.3 cm. diameter). After a few seconds to allow the aliquot to soak in, all discs were dropped into a washing solution of ice-cold 10% trichloroacetic acid. The amount of washing solution in this and subsequent steps was about 5–10 ml. per disc; the time for each step was about 15 min., during which the solution and discs were swirled occasionally. A second 10% trichloroacetic acid wash was done, then five washes in cold 3% perchloric acid, two in cold 95% ethanol, and two in absolute ether. The discs were air-dried, placed in glass vials and counted in a Packard Tri-Carb liquid scintillation counter using a toluene solution of 4 g./l. 2,5-diphenyloxazole and 0.5 g./l. 1,4-bis-2-(5-phenyloxazole)-benzene.

Free radioactivity in the liver homogenates was determined as follows: 1.00 ml. of the homogenate in a clinical centrifuge tube was treated with 1.00 ml. of cold 10% trichloroacetic acid and kept in ice for 15 min. The protein-nucleic acid precipitate was spun down, and 100 μl. of the clear supernatant were pipetted into a glass vial with 10 ml. of Bray's scintillation solution (Bray, 1960) and counted in the Packard scintillation counter. Values for TCA-soluble radioactivity therefore refer to homogenate which has been diluted by a factor of two, whereas the values for protein (TCA-precipitable) radioactivity refer to the undiluted homogenate. Free radioactivity in the blood was determined as for the liver homogenate except smaller volumes were used.

Biochemical analyses

DNA, RNA, and protein analyses following Schneider (1945) were made at three concentrations of the liver homogenates prepared in distilled water. Three clinical centrifuge tubes were prepared containing 0.25, 0.50 and 1.00 ml. of the homogenate. The first two were brought to 1.00 ml. with distilled water and all were treated with 1.00 ml. of cold 10% perchloric acid to precipitate proteins and nucleic acids. After the precipitate was washed with cold 10% perchloric acid and twice with cold 95% ethanol, the nucleic acids were hydrolyzed in 3 ml. 5% perchloric acid at 90° for 15 min. The supernatant was removed and analyzed for total nucleic acids by ultraviolet absorption at 260 mμ, for RNA with orcinol and for DNA with diphenylamine. The protein precipitate was collected on a pre-weighed Millipore filter, air-dried, and weighed. The diphenylamine reaction gave $OD_{600} = 0.364$/mg. Na DNA, based on standard solutions of calf thymus DNA (General Biochemicals). The DNA content of this preparation (as Na DNA, residue weight 331) was found to be 0.66 mg. per mg. of weighed material, as determined from OD_{260} of an acid hydrolysate; the conversion factor $OD_{260} = 30$ for 1 mg./ml. hydrolyzed Na DNA was calculated from extinction coefficients of isolated nucleosides (Beaven *et al.*, 1955). Recovery in the sample preparation was 75% for calf thymus DNA, and DNA values for the liver homogenates were corrected accordingly. Results are based on the slope obtained in a plot of OD_{600} *vs.* concentration of homogenate. A similar plot of OD_{260} for the hot perchloric acid supernatant *vs.* concentration yielded values for total nucleic acid concentration in the homogenate. A standard nucleoprotein consisting of purified *E. coli* K 12 ribosomes, prepared according to Nirenberg (1964), was tested in the procedure. Recovery of the ribosomal RNA was about 75% after precipitation, washing and hydrolysis, compared with direct acid hydrolysis. Total nucleic acid as mg./ml. of liver homogenate was estimated on the basis of this figure and the conversion factor above, and the DNA content was subtracted to obtain RNA. The results in Table III are expressed in mg. per g. wet weight of liver. Measurement of RNA by the orcinol procedure was unsatisfactory due to apparent interference by a liver component which contributed as much as one-half of the total orcinol reading.

Free amino acid analyses were made by the method of Spies (1952) although modification was required because of interfering materials in the whole liver homogenate. The homogenate was first spun at 15,000 *g* for 10 min. in a Sorvall refrigerated centrifuge to remove large particles and debris and then at 105,000 *g* for 3 hr. in a Spinco L2 centrifuge (at 4°) to remove glycogen and ribosomes. The supernatant was carefully withdrawn by pipette, avoiding the lipid layer; proteins were precipitated by addition of 70% perchloric acid to a final concentration of 5%. The precipitate was spun down in a clinical centrifuge and the clear supernatant was neutralized with NaOH and analyzed at two concentrations for the 230 mμ absorption produced by Cu^{++}-amino acid complexes. Each solution was read against a blank consisting of all ingredients except the $CuCl_2$ reagent. All optical density readings were made with a Beckman DU spectrophotometer equipped with a Gilford Model 2000 photomultiplier unit and recorder. A standard solution of L-alanine (Schwarz Laboratories, Inc.) gave a value of $OD_{230} = 3.2$ for a concentration of 1 μm./ml. amino acid in the final solution.

TABLE I

Effect of low temperature acclimation (10°) on protein synthetic capacity of toadfish liver measured in vivo (running sea water aquaria). Incubation time after arterial injection = 15–30 min. Data expressed as cpm in trichloroacetic acid precipitate (on filter paper disc) divided by cpm in trichloroacetic acid-soluble supernatant

Acclimation period Days at 10°	Number of animals*	Temperature of measurement	Incorporation into protein compared to available free radioactivity in liver
0	9	20–22°	0.24 ± 0.15 (S.D.)
3	4	20–22°	0.27 ± 0.15
3	4	10°	0.09 ± 0.04
7	3	20–22°	0.48 ± 0.11

* Average body weight = 240 g.

RESULTS

Table I shows the results of the first series of experiments on the effect of cold acclimation on incorporation of radioactive amino acids into liver protein *in vivo*. The nine control animals included fed and starved (3–7 day) individuals: there were not enough animals to establish a correlation between nutrition and protein synthetic rate. The fish kept at 10° did not eat during this period, although minnows were available in the tank. As shown, three days at 10°

produced no change in liver protein synthesis, when the fish were returned to 20–22° for measurement. When the measurement was made at 10°, the fish showed a low rate of synthesis, as expected for a drop of 10°, in the absence of compensation due to acclimation. The approximate Q_{10} is 2.5, based on the values in Table I and taking the higher temperature as 21°. Values of free radioactivity in the liver supernatants ranged from 500 to 3500 cpm. In two comparable groups the average for fish incubated at 10° was slightly lower (750 cpm) than that for fish incubated at 20° (1050 cpm). Measurements of free radioactivity in the blood correlated well with that in liver for each individual; either of these quantities thus provided a measure of the effectiveness of the injection. To eliminate this factor all the data have been expressed as the ratio of cpm in protein to cpm in the trichloroacetic acid-soluble phase of the liver homogenate. The ratio did not depend on time of incubation between 15 and 30 min., although small changes would have been obscured by the large variation among individuals. Shorter incubations did show time dependence and are not included in Table I. The large scatter in the final data, as indicated by the reported standard deviations, must be attributed to population variability of unknown origin. Hormonal influences on liver metabolism following the stress of handling and injection may play a role. Adrenal and pituitary hormones have been shown to influence levels of C^{14} leucine incorporation into protein in rat liver following a single intraperitoneal injection of the isotope (Reid *et al.*, 1956).

Table II

Effect of low temperature acclimation (10°) on protein synthetic capacity of toadfish liver measured in vivo (static artificial aquaria). Incubation temperature = 23°; time = 20–30 min.

Expt. no.	Days at 10°	Nutritional state	Number of animals	Incorporation into protein compared to available free radioactivity
1	0	Starved 5 days	4	0.21 ± 0.11 (S.D.)
2	0	Fed	3	0.23 ± 0.16
3	3	Starved 4 days	3	0.13 ± 0.03
4	14	Starved 14 days	4	0.37 ± 0.12
5	14	Fed	4	0.30 ± 0.07

After seven days at 10°, as shown in Table I, an increase in protein synthesis is observed upon measurement at 20°. It must be noted that the number of animals is small and the standard deviation in all these measurements rather large; the absolute values must be viewed accordingly. However, analysis by the standard t-test indicated the difference between the 10°-acclimated and control groups to be significant at $P = 0.05$. Thus, the results strongly suggest that liver protein synthesis in toadfish exhibits at least a partial compensation in Precht's (1958) terminology.

The results for *in vivo* protein synthesis in the second series of experiments are given in Table II. In this series more attention was given to nutrition, although again no reliable correlation between feeding and protein synthesis could be established. Fish labelled "fed" had taken minnows one or two days before the experiment; remains were found in the gut at autopsy. The fish kept at 10°

showed little interest in the minnows in the tank, but even after two weeks the toadfish showed no significant weight loss or other apparent sign of starvation. For Expt. 5 the fish were hand-fed by holding minnows in front of them and tapping the mouth until they snapped angrily. The fish were also observed carefully for behavioral changes during 10° acclimation. Unfortunately the toadfish is by nature a sluggish bottom dweller, and the specimens used here conformed to this description equally well at 10° and 20°. Respiratory movements averaged about 8 per min. at 10° and did not change over the two-week acclimation period. The only difference noted was that some of the two-week acclimated fish, after having been warmed to 20°, were hyperactive and difficult to handle during the injection procedure. Effects of low temperature acclimation on oxygen consumption in this species have not been reported, although it would be particularly interesting in view of the unusually high skin respiration of toadfish which permits them to survive 1–2 days out of water (Schwartz and Robinson, 1963).

As indicated in Table II, the two groups of control (23°) fish, fed and 5-day starved, gave comparable values for liver protein synthesis. The fact that all values in the second series are less than in the first is due primarily to the lower efficiency for counting filters of the scintillation counter used for the second series. This, of course, does not affect the results on a comparative basis. Other differences in the second series were the use of static aquaria, and the slightly larger size of the fish collected later in the season. Females in this group had large well-developed eggs. In order to test for a possible effect of temperature shock as a result of transfer from 10° to over 20° immediately before the experiment, a group of fish was again assayed after 3 days at 10°. The average value of 0.13 is low compared to the controls; it is not certain what significance this may have, particularly since it was not observed in the first series. In any case, the sudden transfer of the fish from 10° to 23° does not produce by itself the increase of protein synthesis found after the longer periods at 10°. Expts. 4 and 5 in Table II show the effect of 14 days of 10° acclimation on liver protein synthesis. Comparison of the average of Expts. 4 and 5 with the average of 1, 2 and 3 yields an increased protein synthesis of about 75% as a result of low temperature acclimation (significant at $P = 0.2$ by the t-test). This is in good agreement with the results of Table I.

Biochemical analyses were also performed in the second series to determine if any major alterations of the cellular constituents involved in protein synthesis occurred as a result of 10° acclimation. Of particular importance is the free amino acid pool of liver because the amount of incorporation of the injected isotope will depend on the specific activity of the pool as well as on the rate of protein synthesis. If the pool size does not vary greatly, then the present method of measurement, based simply on cpm per unit volume of homogenate, is justified. On the other hand, if the amino acid pool in cold-acclimated fish is low, then the specific activity will be higher for the same amount of isotope, and incorporation of label into protein will be increased accordingly (this assumes that the rate is not directly dependent on pool concentrations, in which case, the effect would cancel out). As shown in Table III, although there is some decrease in free amino acid concentration in the cold-acclimated fish (Expts. 4 and 5), it is not sufficient to account for the 75% increase in incorporation into protein shown in Table II. In fact, the observation that fish at 10° for 3 days (Expt. 3) show the same low pool value suggests that the decrease may be related to the low temperature treatment but not to the acclimation phenomenon. The present values for free amino acid concentration in a fish liver are similar to those reported for rat liver. The sum of the isolated amino acids of rat liver (one day starved or protein-fed animals) reported by Wiss (see Tarver, 1963) amounts to 24 μmoles/g. liver.

Individual liver-to-body-weight ratios generally showed the expected increase with body weight (Robinson *et al.*, 1960); average ratios in the cold acclimated fish are slightly lower than controls, probably because the fish did not eat during most of the period. This is also indicated by the slight increase of DNA per gram of tissue. Protein yields, however, did not change. Although no analysis was done for lipid and glycogen, it was noted during the centrifugation steps used in preparation for amino acid analysis that the lipid layer formed in the 15,000 *g* centrifugation was almost entirely lacking in cold-acclimated fish and the glycogen pellet obtained at 105,000 *g* was reduced in size. This may account for the slight loss in liver mass in the cold-acclimated animals.

TABLE III

Biochemical analyses for livers of control and cold-acclimated toadfish (incorporation data in Table II)

Expt. no.	Body wgt. average	% Liver	mg./g. tissue (wet)			μm./g. tissue
		body	DNA	RNA	Protein	Free amino acids
1	241	2.8	2.36 ±0.27	3.85 ±1.40	135 ±15	29 ±3
2	314	4.0	2.28 ±0.30	4.15 ±1.30	112 ±6	26 ±3
3	250	3.7	2.26 ±0.25	4.05 ±2.00	116 ±9	23 ±2
4	279	3.1	2.81 ±0.19	5.45 ±1.95	110 ±7	23 ±2
5	260	2.8	2.48 ±0.25	3.80 ±1.95	133 ±17	21 ±2

The values obtained for total RNA per g. liver showed wide variation, as indicated by the large standard deviation. Examination of the individual data revealed that values clustered around two levels; about 6 mg./g. and about 2 mg./g. Averaging of the high and low values separately yielded values of 5.70 ± 0.70 mg./g. for 11 fish and 2.15 ± 0.20 mg./g. for 7 fish. These are both low compared with values obtained by the same method for rat liver (Haschemeyer and Gross, 1967) of about 8 mg./g. in total homogenates. The finding of the higher RNA content for the seven fish which contained eggs suggested a correlation of liver RNA content with the sex of the animal. The levels of all tissue constituents measured differ appreciably from those reported for goldfish liver (Das, 1967), when compared in the same units. In that study, however, the fish were well-fed, with resultant hypertrophy of the liver in the low temperature group and concomitant increase of protein and decrease of

DNA in relation to tissue mass. In spite of the higher rate of protein synthesis, such hypertrophy was not observed in the toadfish liver, probably because the fish ate little on their own initiative during the low temperature period.

DISCUSSION

The purpose of these experiments was to determine what type of compensation might occur in liver protein synthesis of a marine fish, following varying periods at a temperature at the lower end of the animal's normal environmental range. Protein synthesis was assessed in terms of the incorporation of C^{14} amino acids into protein compared to the available free radioactivity in the liver. The procedure involving injection into the arterial blood system permitted rapid uptake of labelled amino acids by the liver; thus incorporation into protein could be measured after short intervals, before degradation or export would be expected to significantly affect the levels of labelled proteins. Rapid pulse measurements are well advised in view of the short half-lives of some liver proteins (in rat), particularly inducible enzymes; tryptophan pyrrolase (peroxidase), for example, is degraded with a half-life of about 2.3 hr. (Feigelson *et al.,* 1959). Other proteins are relatively long-lived; ribosomal protein probably has a half-life of five days, like that of liver ribosomal RNA (Loeb *et al.,* 1965).

The data presented here establish that on the average, toadfish acclimated under laboratory conditions to a temperature of 10° exhibit an increase in liver protein synthesis of about 75% over control fish acclimated to 20–23°, when both are measured at the higher temperature. The biochemical analyses indicated little alteration in tissue components of liver involved in protein synthesis, under the acclimation conditions used in these experiments. Most important, no significant change in free amino acid pools occurred, which would produce some uncertainty in the use of incorporation data as a measure of protein synthesis. Although Das and Prosser (1967), in a study of goldfish acclimation reported during the course of the present work, were unable to establish a significant effect in liver in short-time experiments, this may have been due to the use of intraperitoneal injection. They found, however, that accumulation of label in protein over long periods was increased in cold-acclimated fish, consistent with a greater protein synthetic rate.

The present results support the hypothesis that a control in the protein synthetic pathway may provide a common basis for increased levels of enzymes in respiratory metabolism and other essential pathways responsible for physiological adaptation to low temperatures. Such a change must of course be accompanied by associated changes in degradation rates or rates of protein export from the liver to arrive at a new equilibrium level of proteins. Although a common site for control is suggested by the magnitude of the effect, this does not mean that all proteins are necessarily equally affected; for example, an increase in rate at a step common to all proteins will not affect proteins whose synthesis is rate-limited at another step. The possibility of selective control has been suggested by the finding that different codons are used in the insertion of leucine (Weisblum *et al.,* 1965) and arginine (Weisblum *et al.,* 1967) into the α chain of rabbit hemoglobin. In the case of leucine it was shown by *in vitro* protein synthesis with *E. coli* transfer RNA that a minor species of leucine-accepted tRNA was required to insert leucine into a particular site on the α chain. Thus, the level of a "rare" transfer RNA or its associated aminoacyl-tRNA synthetase may limit the synthesis of some proteins, even during a state of generally increased protein synthesis. Another way in which proteins may be selectively controlled results from special requirements for release from the messenger RNA-ribosome complex or their influence on polyribosome activity. It has been suggested that in hemoglobin the dependence of globin synthesis upon the availability of the prosthetic group is due to a control over release from the protein synthetic site (Gribble and Schwartz, 1965). Another study shows increased size and stability of reticulocyte polyribosomes in response to hemin addition (Grayzel *et al.,* 1966).

Thus, the increased protein synthetic rate in toadfish liver provides a simple mechanism for obtaining increased levels of many proteins, without requiring a specific derepression of genes, as in the Jacob-Monod model (1961) of bacterial enzyme induction. Although the present results refer only to liver, it is quite likely that other tissues exhibit the same phenomenon. Das and Prosser (1967) found evidence for temperature compensation of protein synthesis in gill and muscle tissue of goldfish. Mews (1957) and Jankowsky (1960) have reported increased protein synthesis during cold adaptation of frog skeletal muscle. In order to investigate the possibility of a common translational-type control over protein synthesis, subject to variation with acclimation temperature, we have turned to the study of protein synthesis and the components of the protein synthetic system *in vitro.* These studies will be reported in a separate communication.

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SUMMARY

Liver protein synthesis was measured with a rapid arterial injection procedure in control (20–23°) and cold-acclimated (10°) toadfish. The results were expressed as the cpm of radioactive amino acids incorporated into protein compared to cpm of free radioactivity in the liver homogenate, to correct for variability in injections and in uptake of amino acids by the liver. The results show that, when measured at 20–23°, the 10° acclimated fish possess liver protein synthetic capacity about 75% greater than fish maintained at the higher temperature. Two series of experiments, in which fish were maintained in running sea water aquaria or in static artificial sea water aquaria, gave comparable results. The livers were analyzed for DNA, RNA, protein and free amino acids. The levels of these constituents resembled those in mammalian liver with the exception of RNA, which was lower. Under the conditions of these experiments no significant changes were observed in the constituents measured as a result of two-week cold acclimation.

The possibility of a common translational-type control over protein synthesis to account for increased enzyme levels in cold temperature acclimation is discussed.

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PIGMENT COMPOSITION OF SIPHONALES ALGAE IN THE BRAIN CORAL FAVIA[1]

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Many types of brain corals found on the Great Barrier Reef (*Favia, Porites*, and *Goneastrea*) contain a subsurface layer of a green filamentous alga, which forms a curved zone, 0.5–1.0 cm. wide, some 1–3 cm. beneath the surface layer of brown zooxanthellae. This green algal layer was first observed in corals from the Great Barrier Reef by Marshall and Stephenson (1928), and was found by Odum and Odum (1955) to contribute even more plant biomass to coral communities than the well-known coral zooxanthellae. The studies of Odum and Odum emphasized for the first time the importance of both types of coral symbionts to the productivity of reef communities.

Samples of green layers taken from the brain coral *Favia* were studied in the present work, and were found to consist mainly of the alga *Ostreobium:* probably *Ostreobium Reineckii* Bornet.[2] Preliminary observations on the nature of the algal pigments were made by Drs. L. Muscatine and F. T. Haxo (personal communication), who separated chlorophylls *a* and *b* from the green layer by thin layer chromatography and identified the fractions by absorption spectra. These observations were of interest taxonomicallly, since *Ostreobium* had been placed in the phylum Chrysophycophyta by Scagel (1966), in the family Phyllosiphonaceae within the Chlorococcales by Christensen (1962) and Parke and Dixon (1964), and in the family Phyllosiphonaceae within the Siphonales by Fritsch (1948) and Taylor (1957). The presence of chlorophylls *a* and *b* in the alga *Ostreobium* definitely excluded membership within the Chrysophycophyta, but could not distinguish between the other taxonomic possibilities.

A detailed re-examination of the full complement of photosynthetic pigments in the green layer of the brain coral *Favia pallida* Dana[3] was begun on the basis of these preliminary observations. These studies also formed part of a general study of the physiology and photosynthetic capacity of the deep algal layer (Halldal, 1968; Shibata and Haxo, unpublished data). The pigments were examined by two-dimensional paper chromatography, and identified by R_f values and absorption spectra. The alga in the green layer of the brain coral *Favia* was found to contain the major pigments of the order Spihonales belonging to the class Chlorophyceae.

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[1] Research begun on the University of California Research Vessel R/V "Alpha Helix" during the 1966 Expedition to the Great Barrier Reef, North Queensland, Australia. The shipboard work was supported by the National Science Foundation of the U. S. A. These studies were carried out in collaboration with Drs. F. T. Haxo, P. Halldal, and K. Shibata.

[2] Identified by Dr. W. Randolph Taylor and Dr. M. Nizammudin.

[3] Identified by Dr. E. C. Allison.

METHODS

1. *Extraction of pigments*

(1) *Coral:* Small pieces of coral containing green layers well separated from the zooxanthellae layer were chiselled out from the coral, and freed from any surface brown zooxanthellae. The coral was extracted with methanol for 1–2 hours in the dark in the presence of $MgCO_3$, to prevent possible acidification of the extract during the long extraction period. Several changes of methanol were made until no further pigment was released and the coral layer was colorless. The combined methanol extracts were clarified by centrifugation at 1–2000 *g* for 5 minutes, and the pigments were transferred to diethyl ether by adding an equal volume of ether to the methanol extract and washing once or twice with a volume of 10% NaCl solution 5–10 times that of the methanol + ether extract. All the pigments migrated to the ether layer, which was collected, concentrated to a small volume by evaporation under nitrogen, and used directly for chromatography.

(2) *Algae:* Two other representatives of the order Siphonales (*Halimeda* sp. from Princess Charlotte Bay, North Queensland, and *Codium* sp. from Port Hacking, New South Wales) were studied in order to obtain pigments for comparison with those in the green layer. *Codium* species from the same locality were previously analyzed by Strain (1965). The tissues of the algae were extracted by homogenizing in methanol with added $MgCO_3$, and the extracts prepared for chromatography as above.

2. *Chromatography*

(1) *Solvents:* Solvents used in all cases were A. R. Grade, and were not further purified.

(2) *Paper:* Pigments were chromatographed on Whatman No. 3 (or No. 3 MM) paper, using the two-dimensional solvent system of Jeffrey (1961). This procedure separates the chlorophylls and major carotenoid fractions from each other, but may not fully resolve all carotenoid isomers.

(3) *Thin Layer:* Thin layers of Al_2O_3 and MgO (3:1 w/w) were used to separate α- and β-carotenes. The solvent system was 4% ethyl acetate in hexane (Chapman, 1966). Standard α-carotene for reference was obtained from the cryptomonad *Chloromonas* sp. and β-carotene was isolated from the green flagellate *Dunaliella tertiolecta.* The absorption maxima in petroleum ether (60°–80°) were 476, 448, and 424 for α-carotene, and 482, 451, and 430, β-carotene.

3. *Identification of pigments*

Pigments were identified by R_f values, and by absorption spectra of pigment fractions eluted from paper chromatograms in different solvents. Absorption curves were taken with recording spectrophotometers (Beckman DB, and Unicam SP 700).

4. *Determination of chlorophylls a and b*

Ratios of chlorophylls *a*:*b* were determined in extracts in 90% acetone, using the equations of Humphrey and Jeffrey (in preparation):

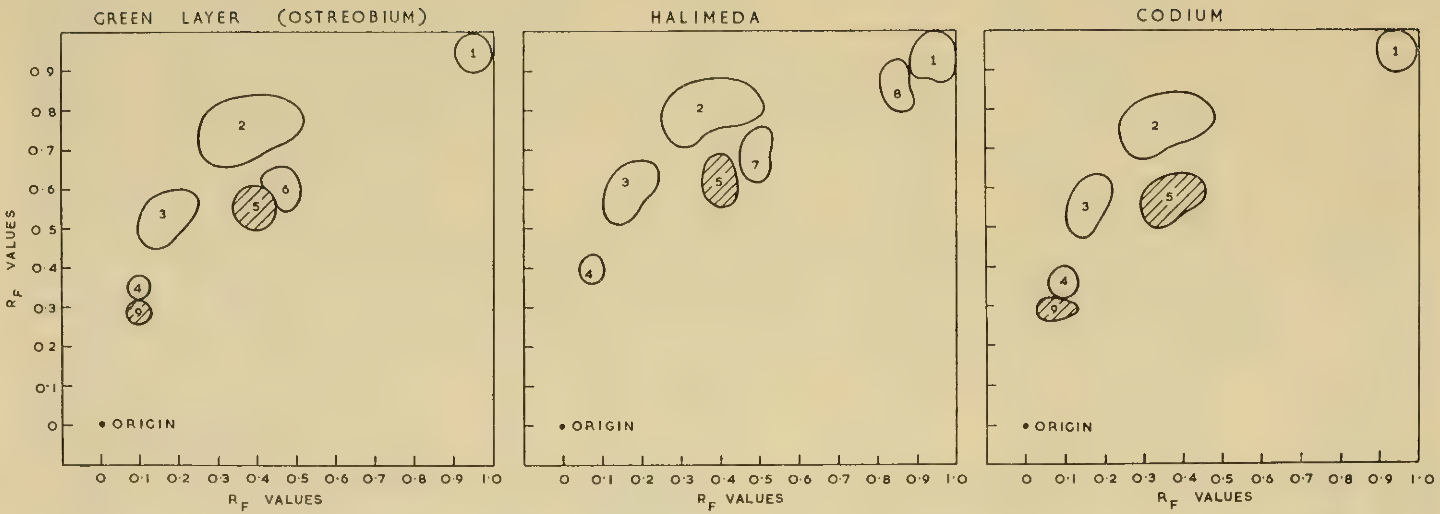

FIGURE 1. Two-dimensional paper chromatograms of pigments from *Halimeda*, *Codium*, and the green layer, *Ostreobium*. 1. Carotenes (yellow). 2. Chlorophyll *a* (blue-green). 3. Chlorophyll *b* (olive-green). 4. Neoxanthin (bright yellow). 5. Siphonein (pink-orange). 6. Unknown (yellow-orange). 7. Violaxanthin (pale yellow). 8. Lutein (yellow). 9. Siphonaxanthin (pink-orange).

$$\text{Chlorophyll } a = 13.50\ E_{663} - 2.91\ E_{645};$$
$$\text{Chlorophyll } b = -4.33\ E_{663} + 21.20\ E_{645};$$

where chlorophyll = concentration of chlorophyll in μg./ml., E = extinction in liters/gm. cm. in a 1-cm. cell.

RESULTS

Figure 1 shows chromatograms of pigments from the green layer (*Ostreobium*) together with those of two-well-known members of the Siphonales, *Halimeda* and *Codium*. Each organism contained chlorophylls *a* and *b*, a carotene zone, several yellow xanthophylls and one or more prominent pink-orange xanthophylls. Table I gives R_f values of the pigments from each alga, with tentative identification. Table II gives absorption maxima of the pigment fractions from each of the three organisms, compared with published maxima of authentic samples. The yellow xanthophylls, lutein, violaxanthin, and neoxanthin, which are normal members of the Chlorophyceae, were identified by R_f values and absorption maxima in *Halimeda,* but only neoxanthin was found in *Codium* and in the green layer. The green layer contained, however, large amounts of an unknown yellow-orange xanthophyll. The two pink xanthophylls, siphonein and siphonaxanthin, which are characteristic of the Siphonales, were found both in *Codium* and in *Ostreobium,* but only siphonein was present in *Halimeda.* It appears that some variations in the full complement of pigments which have been described for the group (Strain, 1958) are possible.

TABLE I

R_f values of pigments from Halimeda, Codium, and the green layer, Ostreobium
Paper chromatography in two solvent systems

Fraction	Pigment	Color	Rf values					
			4 %*n*-propanol/pet. ether			30% $CHCl_3$/pet. ether		
			*Halimeda**	*Codium*	*Ostreobium*	*Halimeda**	*Codium*	*Ostreobium*
1	Carotenes	Yellow	0.95	0.94	0.94	0.94	0.94	0.94
2	Chlorophyll *a*	Blue-green	0.80	0.75	0.74	0.38	0.35	0.36
3	Chlorophyll *b*	Olive-green	0.59	0.55	0.52	0.17	0.15	0.16
4	Neoxanthin	Bright-yellow	0.38	0.36	0.35	0.06	0.09	0.09
5	Siphonein	Pink-orange	0.59	0.58	0.56	0.37	0.37	0.40
6	Unknown	Yellow-orange	—	—	0.61	—	—	0.46
7	Violaxanthin	Pale-yellow	0.64	—	—	0.50	—	—
8	Lutein	Yellow	0.80	—	—	0.86	—	—
9	Siphonaxanthin	Pink-orange	—	0.30	—	—	0.05	—

* *Halimeda* pigments showed slightly higher R_f values in this solvent. It is known that other compounds (*e.g.*, lipids) can force R_f values to higher levels during development (Sestak, 1958). R_f values are therefore not absolute, but give relative orders of separation.

The carotene fraction from the organisms gave one zone on paper chromatography, and showed absorption maxima close to those of α-carotene (Table II). To determine more specifically the presence of α-carotene, extracts were chromatographed on thin layers of alumina/magnesium oxide (3:1) with 4% ethyl acetate in hexane as solvent (Chapman, 1966). In this system, α- and β-carotenes had R_f values of 0.67 and 0.41, respectively. The carotene fractions from *Halimeda,*

TABLE II

Absorption maxima of pigments from Halimeda, Codium, and the green layer, Ostreobium (Fractions separated by paper chromatography)

Fraction	Color	Organism	Absorption maxima (nm)	Solvent	Identification
1	Yellow	*Halimeda*	423,443, 470	diethyl ether	α-carotene
		Codium	417, 445, 475		
		Ostreobium	423, 445, 472		
		Published maxima	422, 445, 475*		
			430, 450, 480*		β-carotene
2	Blue-green	*Halimeda*	426, 661	diethyl ether	Chlorophyll *a*
		Codium	429, 661		
		Ostreobium	429, 661.5		
		Published maxima	430, 662†		
			428.5, 660.5‡		
3	Olive-green	*Halimeda*	453, 643	diethyl ether	Chlorophyll *b*
		Codium	453, 643		
		Ostreobium	455, 644		
		Published maxima	455, 644†		
			452.5, 642‡		
4	Bright yellow	*Halimeda*	414, 437, 464	diethyl ether	Neoxanthin
		Codium	416, 440, 468		
		Ostreobium	trace only		
		Published maxima	414, 437, 466*		
5	Pink-orange	*Halimeda*	460	ethanol	Siphonein
		Codium	462, (457, 481 pet. ether)		
		Ostreobium	465.8		
		Published maxima	465**, (454, 480** pet. ether)		
6	Yellow-orange	*Halimeda*	not present	ethanol	Unknown
		Codium	not present		
		Ostreobium	449, 470.1		
7	Pale yellow	*Halimeda*	415, 439, 468	ethanol	Violaxanthin
		Codium	not present		
		Ostreobium	not present		
		Published maxima	417, 442, 471*		
8	Yellow	*Halimeda*	420, 446, 476	ethanol	Lutein
		Codium	not present		
		Ostreobium	not present		
		Published maxima	420, 446, 476*		
9	Pink-orange	*Halimeda*	not present	ethanol	Siphonaxanthin
		Codium	449, (450, 477 pet. ether)		
		Ostreobium	trace only		
		Published maxima	450, (451, 480** pet. ether)		

* Losev 1964 (including supplementary data of Strain, 1938; and Karrer and Jucker, 1950).
† Smith and Benitez (1955).
‡ Strain, Thomas and Katz (1963).
** Strain (1958).

Codium, and the green layer of *Favia* separated into two zones on the thin layer system: one major fast-running yellow zone corresponding to α-carotene ($R_f = 0.67$), and one minor slower-running orange zone corresponding to β-carotene ($R_f = 0.41$). The presence of α-carotene as the major hydrocarbon carotenoid was therefore established in the three algae by the thin layer technique.

Chlorophyll *a* and *b* ratios were determined in *Halimeda, Codium,* and the green layer. The chlorophyll *b* content was relatively high in the three algae, being two-thirds that of chlorophyll *a* (chlorophyll b:chlorophyll $a = 0.66$, 0.67, and 0.79 in *Halimeda, Codium,* and the green layer, respectively). In higher plants and the green algae chlorophyll *b* is normally one-third that of chlorophyll *a* ($b{:}a = 0.3$).

DISCUSSION

The pigments of representative members of the order Siphonales have been studied by Strain (1958, 1965). He found that algae of this order within the Chlorophyceae contained the normal pigments of the green algae and higher plants —having chlorophylls *a* and *b* and the xanthophylls lutein (with or without zeaxanthin), violaxanthin and neoxanthin. In addition, these algae contained some special carotenoids, namely the pink-orange xanthophylls siphonein and siphonaxanthin, and α-carotene accompanied by small amounts of the β-isomer. The two free-living members of the Siphonales studied here showed this general pattern, although the full complement of xanthophylls expected was not present in either organism. *Halimeda* contained the yellow xanthophylls lutein, violaxanthin and neoxanthin, but only one pink-orange xanthophyll, siphonein, whereas *Codium* possessed both siphonein, siphonaxanthin and neoxanthin, but lacked lutein and violaxanthin. Both algae contained α-carotene as the major carotene, with small quantities of the β-isomer.

The green subsurface algal layer (*Ostreobium*) in the brain coral *Favia* showed a similar carotenoid pattern. α-carotene and siphonein were major components, with traces of siphonaxanthin; of the yellow xanthophylls, only neoxanthin was detected. A prominent unidentified yellow-orange xanthophyll, with absorption maxima at 470 and 449 mm in ethanol, was also present. From the evidence it appears that the filamentous alga *Ostreobium* inhabiting the brain coral *Favia* is appropriately grouped with the Siphonales, without however possessing the full complement of xanthophylls which have been described for the group. Strain (1965) examined 14 members of the Siphonales, and found only one species (*Caulerpa filiformis*) in which the full complement of pigments was not present. In this species both siphonein and siphonaxanthin were missing.

Both *Halimeda, Codium,* and the green layer (*Ostreobium*) contained relatively large amounts of chlorophyll *b,* approaching two-thirds to three-quarters the content of chlorophyll *a.* This is in contrast to other members of the Chlorophyceae and higher plants, where chlorophyll *b* is only one-third that of chlorophyll *a.* A wider survey would be needed to ascertain whether this high proportion of chlorophyll *b* is a characteristic of the Siphonales.

Two samples of the *Favia* green layer were extracted—one from 2–3 cm. below the surface of the coral, and the other from a depth of 6 cm. The first sample showed no trace of chlorophyll decomposition products, indicating that the algae were in a physiologically healthy state. The second sample, taken deep within the coral, showed small zones of chlorophyll decomposition products (pheophytins, chlorophyllides, and pheophorbides). It is evident that in the very deep layers the cells eventually become moribund, with consequent decomposition of the chloroplast pigments.

The pigment evidence provides some guidance to the taxonomic affinities of *Ostreobium.* The presence of chlorophylls *a* and *b* definitely places this alga within the Chlorophyta, and excludes membership within the chlorophyll *c*-containing Chrysophycophyta. Furthermore, *Ostreobium* contains siphonein and siphonaxanthin, two xanthophylls which are found only in members of the Siphonales. On the basis of present evidence, it therefore seems appropriate to group *Ostreobium* within the order Siphonales in the class Chlorophyceae.

SUMMARY

1. The photosynthetic pigments of the green subsurface layer (*Ostreobium*) of
The pigments found were chlorophylls *a* and *b,* α- and β-carotene, siphonein, traces
the brain coral *Favia* were studied by two-dimensional paper chromatography.
of siphonaxanthin and neoxanthin, and an unknown yellow-orange xanthophyll.

2. The pigment composition of *Ostreobium* closely resembled that of two members of the Siphonales, *Halimeda* and *Codium.* Therefore, this alga may be appropriately grouped within the Siphonales.

3. The three algae, *Halimeda, Codium,* and *Ostreobium* contained a high proportion of chlorophyll *b,* from two-thirds to three-quarters that of chlorophyll *a.*

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PHOTOSYNTHETIC PIGMENTS OF SYMBIOTIC DINOFLAGELLATES (ZOOXANTHELLAE) FROM CORALS AND CLAMS[1]

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The brown symbiotic algae, or zooxanthellae, that live in great numbers in the tissues of some marine invertebrates, are considered on morphological evidence to be closely related to the dinoflagellates (Klebs, 1884; Kawaguti, 1944; Pringsheim, 1955). In the animal tissues the cells are in a non-motile vegetative state, without flagella or well-defined girdle, but upon isolation from the host tissue some zooxanthellae develop into typical gymnodinioid swarmers (Zahl and McLaughlin, 1957; McLaughlin and Zahl, 1959). Freudenthal (1962) has studied transformations in great detail in zooxanthellae from the jellyfish *Cassiopea* sp., and he has created a new genus, *Symbiodinium microadriaticum,* within the Dinophyceae, to accommodate this zooxanthella form. Droop (1963), however, has pointed out that "it is by no means certain that all zooxanthellae are dinoflagellates," and McLaughlin and Zahl (1966) warned that "caution must be exercised in the matter of speciation."

Knowledge of the photosynthetic pigment composition of algae can, when taken together with morphological evidence, provide a firm basis for the recognition of group affinities. This biochemical approach becomes increasingly powerful as gaps in our knowledge of the pigment composition of rare as well as known algal groups are filled. Studies of zooxanthellae pigments have, however, been relatively few. Present knowledge of the pigment composition of zooxanthellae rests largely upon studies by Strain, Manning, and Hardin (1944) of the pigment composition of the free-living dinoflagellate *Peridium cinctum* and the endosymbiont of the sea-anemone *Bunodactis xanthogrammica* (now *Anthopleura xanthogrammica; cf.* Ricketts and Calvin, 1962). Using chromatography on columns of powdered sucrose they found that the pigment composition of the zooxanthellae exactly paralleled that of *Peridinium* in possessing chlorophylls *a* and *c*, *β*-carotene, and the xanthophylls peridinin and neo-peridinin, diadinoxanthin and dinoxanthin. It was concluded from the pigment evidence that the *Anthopleura* zooxanthellae and the dinoflagellate *Peridinium* "may belong to the same or related plant groups." However, identity of the former as cryptomonads from morphological evidence could not be excluded at the time. Subsequent biochemical analyses of cryptomonads by Haxo and Fork (1959) and Mallams *et al.* (1967) showed that these algae possess a unique complement of biliproteins and carotenoids, and thus any relationship between zooxanthellae and cryptomonads seems now to be completely excluded.

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[1] Most of the experimental work described in this paper was carried out on board the University of California research vessel, R/V "Alpha Helix," in Princess Charlotte Bay, North Queensland, Australia from July to September, 1966. The Expedition was supported by the U. S. A. National Science Foundation. These studies were carried out in collaboration with Drs. P. Halldal and K. Shibata.

Earlier, Heilbron, Jackson and Jones (1935) crystallized a red-orange pigment from the sea-anemone *Anemonia sulcata* which they called sulcatoxanthin. Strain *et al.* (1944) recognized that sulcatoxanthin was probably identical with peridinin, especially since this anemone is known to contain large numbers of zooxanthellae (Stephenson, 1935).

During the present Expedition[1] a unique opportunity was provided to examine the photosynthetic pigments of the exotic and little studied tridacnid clam zooxanthellae, and an assortment of coral endosymbionts. The pigments were screened by a simple two-dimensional paper chromatographic method (Jeffrey, 1961), which gave reliable separations of the chlorophylls and carotenoids, and which could be used, together with spectral analysis, as a simple aid to the identification of the pigments present. The pigments of the dinoflagellates *Amphidinium* and *Gymnodinium* spp. were used as standard reference material. The zooxanthellae from five species of tridacnid clams, eight zooantharian and alcyonarian corals and one hydrozoan coral were found to have an identical pigment pattern to that of the dinoflagellate *Amphidinium*. The organisms contained chlorophylls *a* and *c*, β-carotene, peridinin and neo-peridinin, dinoxanthin and diadinoxanthin, and three other unidentified xanthophylls present in very small quantities.

TABLE I

Corals used in zooxanthellae survey

CLASS: Anthozoa; *SUB-CLASS*: Zooantharia; *ORDER*: Scleractinia
- i) *Acropora* sp.
- ii) *Seriatopora* sp.
- iii) *Favia* sp.
- iv) *Fungia* (two species)
- v) *Pocillopora* sp.

SUB-CLASS: Alcyonaria; *ORDER*: Coenothecalia
- vi) *Heliopora* sp.

SUB-CLASS: Alcyonaria; *ORDER*: Alcyonacea
- vii) *Xenia* sp. (two species)

CLASS: Hydrozoa; *ORDER*: Milleporina
- viii) *Millepora* sp.

MATERIALS AND METHODS

MATERIALS

Clams and corals were collected from the Great Barrier Reef just outside Princess Charlotte Bay, North Queensland. Species of tridacnid clams studied were *Tridacna crocea, Tridacna gigas, Tridacna squamosa, Tridacna deresa, Tridacna maxima,* and *Hippopus hippopus.* Identifications were made according to the descriptions provided by Rosewater (1965). Nine corals from both major classes and sub-classes were studied (Table I).

METHODS

A. *Preparation of zooxanthellae*

Zooxanthellae were isolated from clam mantle tissue and corals according to methods worked out by Muscatine (1967).

1. *Clams.* Mantle tissue containing zooxanthellae was excised, and freed as much as possible from supporting muscle tissue. The mantle was rinsed several times in filtered sea water, and cut into small pieces. The tissue was homogenized in sea water in a Waring Blendor for one minute, and the resulting suspension was filtered through six layers of cheesecloth, to free the cells from tissue debris. The dark brown suspension of algal cells was washed three times by centrifuging in filtered sea water at about 2500 *g* for five minutes. The cells were finally resuspended in sea water to a homogeneous suspension. Packed cells could be stored frozen without pigment deterioration for several weeks.

2. *Corals.* Zooxanthellae from hard corals were released by crushing the tissue into small pieces in aluminium foil with hammer or pliers, and rinsing continuously in sea water to wash out the cells. The suspension was filtered through cheesecloth, and the cells washed as before by centrifuging 2–3 times in filtered sea water.

Zooxanthellae from the tentacles of soft corals (*Xenia* spp.), and from the tentacles of *Fungia,* were collected by excising the tentacles and grinding them gently in sea water in a glass Potter-Elvehjem homogeniser. The zooxanthellae, which were released intact by this treatment, were filtered through cheesecloth, and washed by centrifugation as above. In every case, beautiful, clean suspensions of undamaged zooxanthellae were obtained.

B. *Preparation of pigment extracts*

Zooxanthellae were extremely difficult to extract in either acetone or methanol at room temperature. Pigments were readily released, however, if the packed cells were first suspended in a little distilled water, and frozen for 4–8 hours before methanol extraction. This procedure apparently weakened the tough cell wall, and allowed subsequent extraction to proceed readily, without alteration of the pigments. The cells were extracted about 2–3 times in methanol, until the residue was colorless. The methanol extract was clarified by centrifugation, mixed with an equal volume of diethyl ether, and shaken once or twice with a volume of 10% NaCl solution 5–10 times that of the methanol-ether extract. This saline washing caused the pigments to migrate to the ether layer, and methanol and methanol-soluble impurities were washed out in the aqueous phase. The ether layer was then concentrated for chromatography by evaporation under a stream of nitrogen. The ether extracts could be stored in the deep freeze for several days without deterioration of the pigments.

Some of the hard corals were extremely difficult to crush for the collection of zooxanthellae. Small pieces of these corals were extracted "whole" with methanol for several hours at room temperature, in the presence of a little $MgCO_3$, to prevent acidification. Extraction was continued until the coral skeleton was colorless. Pigments were not damaged by this treatment, as was shown by subsequent chromatography.

The dinoflagellates, *Amphidinium* and *Gymnodinium*, were used as standard sources of dinoflagellate pigments. *Gymnodinium* was used mainly as a source of standard dinoxanthin, since in *Amphidinium* the amount of this pigment was extremely small. Cultures were grown in Medium f (Guillard; see Grant, 1967) for about two weeks at 5000 lux. The cells were harvested by continuous centrifugation, and pigments were readily extracted from the packed cells with small volumes of 90% acetone. After 2–3 extractions the residue was colorless. Pigments were transferred from acetone to ether as above, and used directly for chromatography. Samples of *Amphidinium* cells were freeze-dried, for dispatch from Cronulla to Princess Charlotte Bay. The dry powder could be stored in the dark at room temperature for several weeks, without deterioration of the pigments.

C. *Paper chromatography*

Pigment extracts were chromatographed on 22-cm. squares of Whatman No. 3 (or No. 3 Mm paper in two dimensions, according to the method of Jeffrey (1961). Solvents used were AR Grade, and were not further purified. Solvent systems used were 4% *n*-propanol in light petroleum (60–80° C.) for the first dimension, and 30% $CHCl_3$ in light petroleum for the second dimension. Absorption spectra of pigment fractions were obtained by running a number of chromatograms, and eluting the pigment spots. Absorption spectra were taken with a Beckman DB spectrophotometer (for work done on the "Alpha Helix"), a Unicam SP 700 (for work done subsequently at Cronulla), and a Carey Model 14 (for work done subsequently at La Jolla). The latter instruments were calibrated with Hg lines and were considered accurate to 1 nm.

D. *Quantitative determination of pigments*

1. *Spectrophotometric*. Routine quantitative determinations of chlorophylls *a* and *c* were carried out on pigment extracts, either in methanol, 90% acetone, or in diethyl ether. Extinctions were measured at the red maxima of the two chlorophylls, and the concentration calculated from the provisional equations of Humphrey and Jeffrey (in preparation).

In ether:

$$\left[\begin{array}{l}\text{chl } a = 10.5\,E_{662} - 1.0\,E_{628}\\ \text{chl c} = -8.1\,E_{662} + 64.3\,E_{628}\end{array}\right.$$

In 90% acetone:

$$\left[\begin{array}{l}\text{chl } a = 13.31 \times E_{663} - 0.27 \times E_{630}\\ \text{chl } c = -8.37 \times E_{663} + 51.72 \times E_{630}\end{array}\right.$$

In methanol:

$$\left[\begin{array}{l}\text{chl } a = 13.8\,E_{668} - 1.3\,E_{635}\\ \text{chl } c = -14.1\,E_{668} + 67.3\,E_{635}\end{array}\right.$$

where chl = concentration of chlorophyll in ug./ml. and E = extinction in liters/gm. cm. in a 1-cm. cell.

2. *Chromatographic*. The percentage composition of each carotenoid fraction was determined by running several chromatograms simultaneously, and eluting the spots immediately in the appropriate solvent. Solutions were made up to a

measured volume, centrifuged to remove any paper fibers, and the extinctions read without delay. In this, and in all the chromatographic work, operations were carried out in dim light, or in complete darkness. Photo-decomposition of pigments was thus kept at a minimum.

Concentrations of pigments were calculated using the following extinction coefficients:

Chlorophyll *a* in acetone	90 l./gm.cm.	Vernon (1960)
Chlorophyll *c* in methanol	15.2 l./gm.cm.	Jeffrey (1963)
Carotene in ether	250.5 l./gm.cm.	Goodwin (1955)
Peridinin in ethanol	132.5 l./gm.cm.	present work

The concentrations of all other carotenoids, whose extinctions were unknown, were calculated using the extinction coefficient of carotene.

E. *Thin layer chromatography*

Since paper chromatography does not resolve carotene isomers, thin layers of $Al_2O_3 + MgO$ (3:1 w/w) were used to characterize the carotene fractions. Using as solvent 4% ethyl acetate in hexane (Chapman, 1966), α- and β-carotenes were separated with R_f values of 0.67 and 0.41. α-Carotene for reference was obtained from the cryptomonad *Chloromonas* sp., and β-carotene from the green flagellate, *Dunaliella tertiolecta.*

The pink-orange xanthophyll (fraction 9), which remained at the origin in the paper chromatographic system was separated from chlorophyll *c* by chromatography of the "origin material" on thin layers of polyethylene, using 90% acetone as solvent. The pink xanthophyll ran just behind the solvent front ($R_f = 0.95$) with chlorophyll *c* at R_f values of 0.3 and 0.4. The xanthophyll was eluted with ethanol, for spectral analysis.

A thin layer chromatography system, which completely duplicated the paper chromatography method, and which gave even better resolution of all the dinoflagellate pigments, was subsequently developed after returning from the Expedition. This method (to be described in detail in a separate communication) uses plates of specially prepared sucrose, with 0.6% *n*-propanol in petroleum ether (60–80° C.) and 12% chloroform in petroleum ether, as the two-dimensional solvent system. Extracts of *Amphidinium,* and extracts of zooxanthellae from the clam *Tridacna crocea* and the coral *Pocillopora* were chromatographed using this system.

F. *Crystallization of peridinin*

Approximately 6 mg. crystalline peridinin were isolated from *Tridacna gigas* as follows. The mantle tissue was excised from a healthy giant clam, freed substantially of colorless animal tissue and immediately deep frozen. The frozen zooxanthellae-laden mantle tissue weighing some two Kg. was chopped into small pieces and exhaustively extracted in the cold with 95% ethanol. The total pigment was transferred to benzene with the addition of saturated NaCl solution, and the washed and dried pigment solution evaporated to dryness *in vacuo* at 30° C. The pigment was dissolved in 80% aqueous methanol, and the initial separation of the peridinin from other pigments and colorless contaminants was made by passage of the methanolic solution through powdered polyethylene. The peridinin fraction was recovered as the leading zone. Crude peridinin from several such columns was transferred to benzene and then chromatographed on columns of powdered $CaCO_3$, developed with 1–2% acetone in benzene, according to the method of Pinckard *et al.* (1953). Prior to crystallization, the almost pure peridinin was rechromatographed on thin layer plates of silica gel G employing 30% acetone in hexane as developing solvent.

Crystallization of peridinin was carried out according to the procedures of Pinckard *et al.* (1953), once from benzene-hexane and once from ether-hexane mixtures. The homogeneous microcrystalline product was scarlet to warm reddish brown in color, disc-shaped and free of colorless contaminants. After drying *in vacuo* at 65.5° C. the product weighed a little over 6 mg.

The crystals were readily soluble in methanol, ethanol, acetone, ether, and benezene, and virutally insoluble in hexane. Methanolic solutions were found to be strongly hypophasic. The phase distribution in the system 65% MeOH/hexane was 96:4.

TABLE II

Extinction coefficients of peridinin isolated from Tridacna gigas

Solvent		Maxima (nm)	Extinction coefficient $E_{1cm.}^{1\%}$
Acetone	(single broad maximum)	466	1340
90% Acetone	(single broad maximum)	469	1330
Methanol (absolute)	(single broad maximum)	469	1360
Ethanol (absolute)	(single broad maximum)	472	1325
Pyridine	(single broad maximum)	475	1180
Diethyl ether		454,* 475	1450
Benzene		467,* 494	1290
Hexane-ether (9:1)		454,* 483	1470
Chloroform		470,* 490	1290
Hexane		454, 484	—

* Extinctions taken at these maxima.

The melting point of the crystals was determined in capillaries sealed under N_2 employing a Thomas Hoover capillary melting point apparatus. Averaged values for two determinations were: softened at 130.7° C. (cor.) and complete melt at 134.9° C. (cor.). These values agree well with the total melt point of 130° C. reported by Heilbron *et al.* (1935) for sulcatoxanthin (*i.e.*, peridinin) from *Anemonia sulcata.*

For estimation of the extinction coefficient 1.040 mg. peridinin were dissolved in 200 ml. acetone and spectral absorption determined in a 1-cm. path length cuvette in the Cary 14 Spectrophotometer. From this measurement the $E_{1\,cm.}^{1\%}$ at 466 mμ, the wave-length of maximum absorption in acetone, was calculated to be 1340. The ash content of the sample is not known and the extinction coefficient is considered to be minimal. This value is substantially higher than the extinction coefficient of 840 (acetone) reported by Parsons and Strickland (1963) for peridinin from *Amphidinium carterei.* Relative extinctions of peridinin in other solvents were determined by evaporating aliquot samples of the above acetone solution to dryness in a rotary evaporator *in vacuo* at room temperature and dissolving in the appropriate solvent. Extinction values for peridinin in different solvents, and absorption maxima, are given in Table II.

By way of further characterization of peridinin from *Tridacna gigas,* iodine-catalyzed trans-cis isomerizations similar to those performed by Pinckard *et al.*

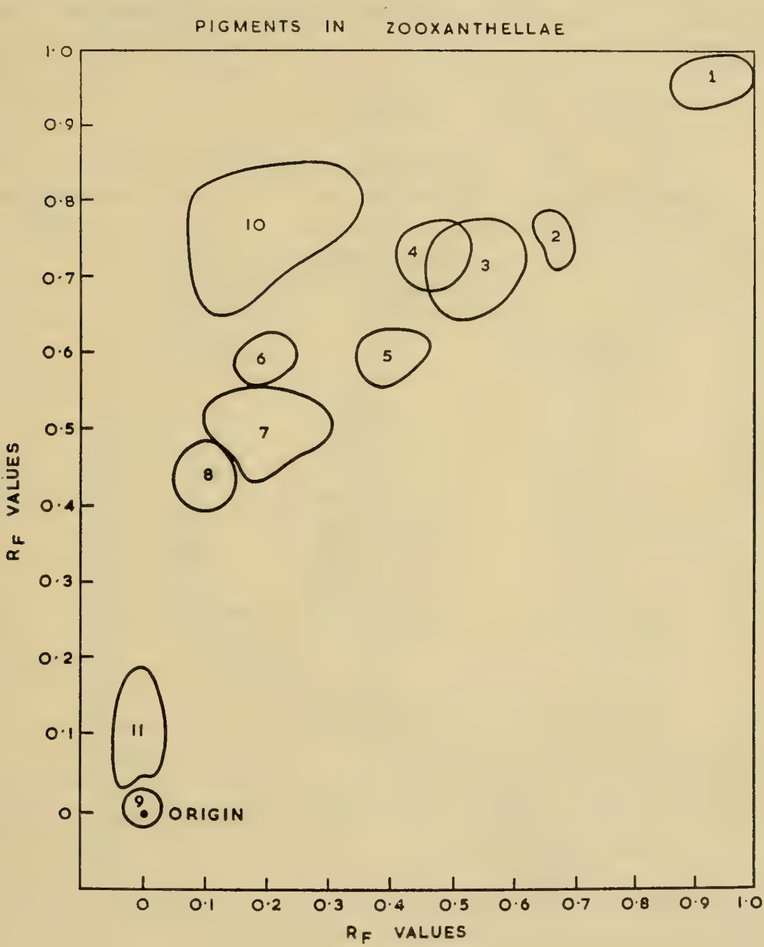

FIGURE 1. Two-dimensional paper chromatogram of pigments in zooxanthellae from *Tridacna crocea.* Chromatographic solvent systems: 1st dimension, 4% *n*-propanol in petroleum ether (60–80°); 2nd dimension, 30% chloroform in petroleum ether. 1, β-carotene (orange). 2, Unknown (pale orange). 3, Diadinoxanthin (yellow). 4, Dinoxanthin (yellow). 5, Unknown (yellow). 6, Neo-dinoxanthin (yellow). 7, Peridinin (brick red). 8, Neo-peridinin (brick red). 9, Unknown (pink orange). 10, Chlorophyll *a* (blue green). 11, Chlorophyll *c* (light green).

(1953) for crystalline peridinin isolated from *Prorocentrum micans* were carried out. In all respects examined the properties of the two preparations were identical, *e.g.*, spectra before and after iodine catalysis of benzene solutions; number, adsorption position and spectral properties of isomers separable on $CaCO_3$ columns; and percentage composition of an equilibrium mixture of stereo-isomers (57% all-trans and 43% combined cis-isomers).

For comparison with one of the original sources used by Strain *et al.* (1944), peridinin was isolated from zooxanthallae from the sea anemone *Anthopleura xanthogrammica.* (Live sea anemones were collected intertidally at La Jolla.) Peridinins from the sea anemone and *Tridacna gigas* were found to be chromatographically homogeneous in the thin layer system, silica gel G-30% acetone in hexane.

RESULTS

Figure 1 shows a typical two-dimensional paper chromatogram of the pigments of zooxanthellae from *Tridacna crocea.* Identical chromatograms were

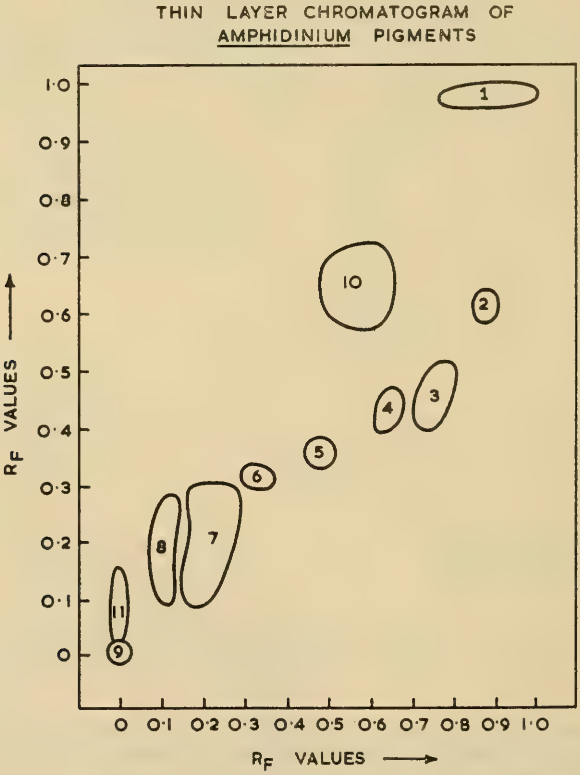

FIGURE 2. Sucrose thin layer chromatogram of pigments in the dinoflagellate *Amphidinium* sp. Chromatographic solvent systems; 1st dimension, 0.6% *n*-propanol in petroleum ether (60–80°); 2nd dimension, 12% chloroform in petroleum ether. Pigment fractions as in Figure 1.

also obtained from the other four clam zooxanthellae, the nine coral zooxanthellae, and the dinoflagellate *Amphidinium*. In every case chlorophyll *a* was accompanied by chlorophyll *c*, and the major carotenoids were always β-carotene, peridinin, neo-peridinin, dinoxanthin, and diadinoxanthin. Average R_f values for dinoflagellate pigments in the two solvent systems are given in Table III. The carotene fraction was shown to consist only of the β-isomer by chromatography of the carotene fraction from zooxanthellae and *Amphidinium* on thin layers of alumina and magnesium oxide.

Figure 2 shows a typical chromatogram of dinoflagellate pigments separated on specially prepared thin layers of powdered sucrose. Again, identical patterns of pigments were obtained with *Amphidinium* and zooxanthellae extracts from the clam *Tridacna crocea* and the coral *Pocillopora*. Since the thin layer method will be described in detail in a separate communication, only results obtained with the paper method, which was used on the Expedition, will be presented here.

TABLE III

R_f values of pigment fractions from zooxanthellae and Amphidinium using paper chromatography

Fraction	Pigment	Color	R_f Values	
			4% *n*-propanol in pet. ether	30% $CHCl_3$ in pet. ether
1	Carotene	Orange	0.96	0.93
2	Unknown	Pale orange	0.75	0.67
3	Diadinoxanthin	Yellow	0.71	0.54
4	Dinoxanthin	Yellow	0.73	0.48
5	Unknown	Pale yellow	0.59	0.40
6	Neo-dinoxanthin	Pale yellow	0.59	0.19
7	Peridinin	Brick red	0.49	0.19
8	Neo-peridinin	Brick red	0.43	0.10
9	Unknown	Pink-orange	0	0
10	Chlorophyll *a*	Blue-green	0.76	0.21
11	Chlorophyll *c*	Light green	0.10	0

Absorption spectra of all pigment zones were analyzed in detail in zooxanthellae from the two clams *Tridacna crocea* and *Hippopus hippopus*, the coral *Pocillopora* and the dinoflagellate *Amphidinium*. The absorption maxima are listed in Table IV, and are compared with absorption maxima of pigments from the dinoflagellate *Peridinium cinctum* given by Strain, Manning and Hardin (1944).

Absorption properties of peridinin from *Tridacna gigas* and *Amphidinium* were checked on more accurate spectrophotometers after the Expedition. The values for peridinin in ethanol obtained on the Expedition with the Beckman DB spectrophotometer were from 473–475 nm (Table IV). Using the same paper chromatography method, the maximum of peridinin in ethanol from *Amphidinium* was 474 nm (Unicam SP700), and from *Tridacna gigas* was 473 nm (Carey Model 14). The absorption maximum of *Tridacna gigas* peridinin prepared as an all-trans sample and then crystallized, was 473 nm; prepared as above but rechromatographed on $CaCO_3$ to obtain the fresh all-trans fraction was 472 nm.

TABLE IV

Absorption spectra of pigments from zooxanthellae of corals, clams, and dinoflagellates

Fraction	Organism	Absorption maxima (nm)	Solvent	Identification
Fraction 1 (orange)	*Tridacna crocea*	426, 448, 475	diethyl ether	*β-Carotene*
	Hippopus hippopus	428, 449, 475	diethyl ether	
	Pocillopora sp.	428, 448, 475	diethyl ether	
	Amphidinium sp	429, 450, 475	diethyl ether	
		429, 450, 478	hexane	
	*Peridinium cinctum**	not given		
Fraction 2 (pale orange)	*Tridacna crocea*	429, 450, 476	ethanol	*Unknown*
	Hippopus hippopus	429, 450, 478	ethanol	
	Pocillopora sp.	427, 450, 477	ethanol	
	Amphidinium sp.	429, 452, 477	ethanol	
	*Peridinium cinctum**	not present		
Fraction 3 (yellow)	*Tridacna crocea*	425, 447, 477	ethanol	*Diadinoxanthin*
	Hippopus hippopus	426, 448, 477	ethanol	
	Pocillopora sp.	425, 447, 476.5	ethanol	
	Amphidinium sp.	425, 448, 478	ethanol	
	*Peridinium cinctum**	448, 478	ethanol	
Fraction 4 (yellow)	*Tridacna crocea*	418, 442, 470	ethanol	*Dinoxanthin*
	Hippopus hippopus	418, 441, 468	ethanol	
	Pocillopora sp.	418, 441, 469	ethanol	
	Amphidinium sp.	418, 443, 472	ethanol	
	*Peridinium cinctum**	441.5, 471	ethanol	
Fraction 5 (pale yellow)	*Tridacna crocea*	408, 427, 455	ethanol	*Unknown*
	Hippopus hippopus	410, 428, 455	ethanol	
	Pocillopora sp.	408, 427, 455	ethanol	
	Amphidinium sp.	408, 427, 455	ethanol	
	*Peridinium cinctum**	not present		
Fraction 6 (pale yellow)	*Tridacna crocea*	425, 441, 467	ethanol	*Neo-dinoxanthin*
	Hippopus hippopus	425, 440, 466	ethanol	
	Pocillopora sp.	420, 440, 465	ethanol	
	Amphidinium sp.	422, 440, 467	ethanol	
	*Peridinium cinctum**	438, 466	ethanol	
Fraction 7 (brick red)	*Tridacna crocea*	475	ethanol	*Peridinin*
	Hippopus hippopus	473	ethanol	
	Pocillopora sp.	475	ethanol	
	Amphidinium sp.	475	ethanol	
	*Peridinium cinctum**	475	ethanol	

* Data for *Peridinium cinctum* taken from Strain, Manning, and Hardin (1944).

† Present, but insufficient for spectral analysis.

TABLE IV—*Continued.*

Fraction	Organism	Absorption maxima (nm)	Solvent	Identification
Fraction 8 (brick red)				
	Tridacna crocea	468	ethanol	
	Hippopus hippopus	465	ethanol	*Neo-peridinin*
	Pocillopora sp.	465	ethanol	
	Amphidinium sp.	467	ethanol	
	*Peridinium cinctum**	464	ethanol	
Fraction 9 (pink-orange)				
	Tridacna crocea†			
	Hippopus hippopus†			*Unknown*
	Pocillopora sp.†			
	Amphidinium sp.	460–466	ethanol	
	*Peridinium cinctum**	not present		
Fraction 10 (blue-green)				
	Tridacna crocea	409, 428, 661	ether	
		410, 428, 662	acetone	*Chlorophyll a*
	Hippopus hippopus	409, 428, 663	ether	
	Pocillopora sp.	409, 428, 663	acetone	
	Amphidinium sp.	408, 428, 660	ether	
	*Peridinium cinctum**	not given		
Fraction 11 (light green)				
	Tridacna crocea	448, 582, 634	methanol	
	Hippopus hippopus	448, 583, 634	methanol	
	Pocillopora sp.	448, 584, 634	methanol	*Chlorophyll c*
	Amphidinium sp.	451, 584, 635	methanol	
	*Peridinium cinctum**	not given		

TABLE V

Percentage composition of carotenoids in zooxanthellae from Tridacna crocea, and the dinoflagellate Amphidinium sp.

Fraction number	Pigments	% of Total carotenoids	
		Tridacna crocea	*Amphidinium* sp.
1	β-carotene	3.0	2.5
2	Unknown	1.5	1.7
3 + 4	Diadinoxanthin and dinoxanthin	12.6	10.5
5	Unknown	1.3	0.3
6	Neo-dinoxanthin	3.1	0.6
7 + 8	Peridinin and neo-peridinin	77.0	84.0
9	Unknown	1.5	0.4
		Total 100.0	100.0

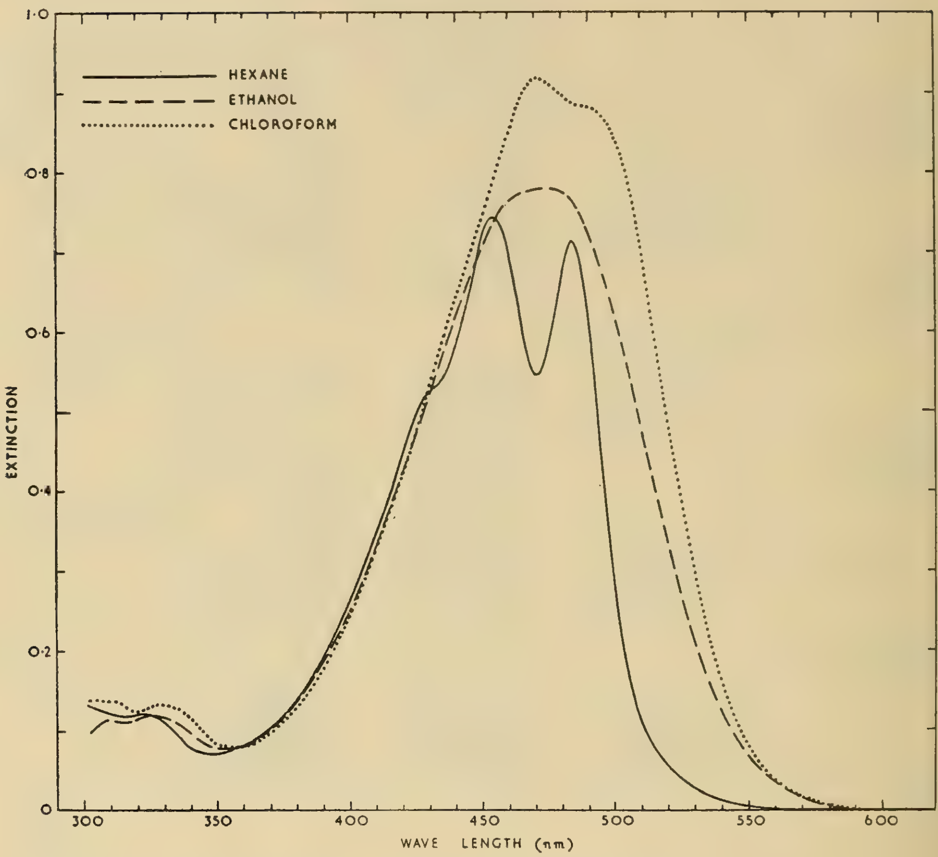

FIGURE 3. Absorption spectra of all-trans peridinin isolated from *Tridacna gigas.*

Peridinin apparently isomerizes fairly rapidly and crystallization of the all-trans fraction may not have excluded traces of the cis-isomers. Cis-trans isomerization of peridinin in benzene solution with iodine caused the maxima to shift from 496 and 468 nm (all-trans) to 493 and 466 nm (equilibrium mixture) after 10 minutes. There was no further spectral change at 120 minutes.

Full absorption curves of peridinin in ethanol, chloroform and hexane are shown in Figure 3. This peridinin was a freshly prepared all-trans fraction from *Tridacna gigas* mantle tissue. Peridinin isolated by paper chromatography from *Pocillipora* zooxanthallae gave similar absorption curves in the three solvents.

The relative proportions of the carotenoids were analyzed by paper chromatography in zooxanthellae from *Tridacna crocea* and in the dinoflagellate *Amphidinum.* Table V shows that in both cases the major xanthophyll fraction, peridinin and neo-peridinin, constituted some 77–84% of the total carotenoids. The other xanthophyll fractions were, by contrast, only a few per cent of the total.

The relative concentrations of chlorophylls *a* and *c* in the zooxanthellae are given in Table VI. In coral zooxanthellae, the content of chlorophyll *c* was about one-tenth that of chlorophyll *a*. In zooxanthellae from clams, and in the dinoflagellates *Amphidinium* and *Gymnodinium,* the chlorophyll *c* content approached two-thirds that of chlorophyll *a*.

No animal pigments could be detected in the clam mantle tissue itself, identical chromatograms and identical absorption spectra being obtained both with extracts of whole mantle tissue and with isolated zooxanthellae. However, R_f values of the dinoflagellate pigments extracted directly from the mantle were always a little higher than those from zooxanthellae processed separately. That the increased R_f values were due to animal tissue factors extracted with the zooxanthellae was indicated by the effect of adding colorless methanol-acetone extracts of white mantle muscle (without zooxanthellae) to extracts of isolated zooxanthellae cells. R_f values of all pigment fractions became increasingly higher with increasing amounts of animal extract present. This phenomenon, of the presence of colorless factors (probably lipids) influencing the R_f values of photosynthetic pigments, is well documented for other plant pigment systems (Sestak, 1958). Consistent R_f values were, however, always obtained from zooxanthellae which were first separated from the mantle tissue before extraction.

TABLE VI

Ratio of chlorophyll c:chlorophyll a in zooxanthellae from corals and clams

	Organism	Chlorophyll *c*:*a*
Corals	*Pocillopora* sp.	0.1
	Fungia sp.	0.07
	Acropora sp.	0.2
	Millepora sp.	0.06
Clams	*Tridacna crocea*	0.6
	Tridacna gigas	0.3
	Hippopus hippopus	0.6
Dinoflagellates	*Amphidinium* sp.	0.6
	Gymnodinium sp.*	0.8

* Taken from Jeffrey (1963).

DISCUSSION

Strain, Manning and Hardin (1944) were the first to study in detail the photosynthetic pigments of the free-living dinoflagellates. Using cultures of *Peridinium cinctum,* and separating the pigments on columns of powdered sugar, the components found were chlorophylls *a* and *c*, β-carotene, a major brick-red xanthophyll peridinin and its neo-isomer, and four minor yellow xanthophylls, diadinoxanthin, dinoxanthin, neo-diadinoxanthin, and neo-dinoxanthin. These pigments were also found in the dinoflagellates *Amphidinium carteri* (Parsons, 1961) *Prorocentrum micans* (Pinckard *et al.*, 1953) and *Gymnodinium* (Jeffrey, 1961) and in zooxanthellae from the sea anemone *Anthopleura xanthogrammica* (Strain, Manning and Hardin, 1944).

Peridinin and dinoxanthin are specific to the dinoflagellates, and have not been found in any other classes of algae. The other pigments (chlorophylls *a* and *c*, β-carotene and diadinoxanthin) are not by themselves taxonomically definitive pigments, since they also occur in other algal groups (Strain, 1958). In the present survey of photosynthetic pigments in zooxanthellae from corals and clams, peridinin was found in every organism tested, and this compound had the same chromatographic and absorption properties as peridinin isolated from the dinoflagellate, *Amphidinium*. In addition, dinoxanthin was found as a minor pigment in all zooxanthellae and dinoflagellate preparations, together with the full complement of chlorophylls and carotenoids originally described by Strain, Manning and Hardin (1944) for *Peridinium cinctum*. The absorption maxima of the pigment fractions from different zooxanthellae corresponded closely to each other and to the published maxima (Table IV). Where small discrepancies were present low extinctions were combined with broad peaks, with consequent difficulties in obtaining accurate maxima. Identification of pigment fractions from their spectral properties was, however, strengthened by the identical R_f values and chromatographic patterns which were obtained.

Peridinin isolated by paper chromatography showed absorption maxima closely similar to freshly prepared all-trans crystalline peridinin. The absorption maxima of peridinin in ethanol from *Tridacna gigas* were at 473 nm (paper chromatography), 473 nm (crystalline "all-trans"), and 472 nm (freshly prepared all-trans), using the Carey spectrophotometer in all cases. With the Beckman DB spectrophotometer, the range of values for peridinin isolated by paper chromatography was 473–475 nm (Table IV). Cis-trans isomerization studies in benzene solution showed that the all-trans fraction had absorption maxima at higher wave-lengths than the individual cis-isomers, or the equilibrium mixture.

The extinction coefficient in acetone of crystalline peridinin from *Tridacna gigas* was substantially higher than that obtained by Parsons and Strickland (1963) for peridinin isolated from *Amphidinium carterei*. The melting point, however, agreed well with the melting point of sulcatoxanthin (peridinin) isolated from the sea anemone, *Anemonia sulcata* (Heilbron *et al.*, 1935). Further, the isomerization behavior of *Tridacna gigas* peridinin resembled similar preparations obtained from *Prorocentrum micans* (Pinckard *et al.*, 1953).

In addition to the major dinoflagellate pigments described, three new xanthophylls were also found in extracts of zooxanthellae and *Amphidinium*. These were readily distinguished both on paper chromatograms and on the sucrose thin-layer plates. Absorption maxima of these fractions taken in ethanol did not appear to correspond to any previously described xanthophyll. Due to the very small quantities present, and the limited duration of the Expedition, it was not possible to analyze these pigment fractions further. Strain, Manning and Hardin (1944) noted a "flavoxanthin-like" xanthophyll in some of their fractions in amounts too small to be analyzed. This may correspond to one or more of the minor xanthophylls described here. Neodiadinoxanthin, found by Strain, Manning, and Hardin (1944) in *Peridinium cinctum,* could not be located in any of the present preparations studied.

Quantitative data on the carotenoid composition of zooxanthellae and *Amphidinium* showed that the major xanthophyll fraction, peridinin and neoperidinin, constituted some 77–84% of the total carotenoids. Diadinoxanthin was always more prominent than dinoxanthin, and together the concentration was about 10–12%, whereas the other xanthophylls were present in very small quantities which varied from 0.3% to 3.0% of the total. Peridinin in these zooxanthellae (Halldal, 1968; Shibata and Haxo, unpublished data), as well as peridinin from *Gonyaulax polyedra* (Haxo, 1960) appears to have an accessory pigment function in photosynthesis, but the function of the minor carotenoid components, if any, is unknown.

The ratios of chlorophylls *a* and *c* varied depending on whether the zooxanthellae came from a clam or coral host. Clam zooxanthellae resembled the free-swimming dinoflagellates in having a chlorophyll *c* content almost two-thirds that of chlorophyll *a,* whereas in coral zooxanthellae the chlorophyll *c* content was only about one-tenth that of chlorophyll *a.* Further studies would be needed to ascertain whether this was a real difference between clam and coral zooxanthellae. Burkholder and Burkholder (1960) analyzed the chlorophyll content of some alcyonarian corals by spectrophotometric methods and found that chlorophyll *c* was equal to chlorophyll *a.* Using more accurate extinction coefficients the chlorophyll *c* was probably about one-half that of chlorophyll *a.*

No chlorophyll degradation products were ever present on chromatograms of freshly prepared zooxanthellae extracts. This suggests that not only were the extraction and chromatographic procedures reliable, but that the zooxanthellae populations were in a healthy state. Zooxanthellae breakdown must therefore occur at some other locus in the animal host than that sampled, or senescent cells may be discharged from the host tissues and do not accumulate. Traces of pheophytins, chlorophyllides and pheophorbides, indicating the presence of senescent cells, would have been readily detected on the paper chromatograms, had they been present.

Zooxanthellae were further indicated to have dinoflagellate affinities by the presence of gymnodinioid swarmers in suspensions of zooxanthellae which were left overnight in sea water. Although these were not studied in detail, they clearly resembled the swarmers described for *Cassiopea* zooxanthellae, by Freudenthal (1962).

The two-dimensional paper chromatography method provided a simple but useful screening technique for determining the pigment composition of a large range of zooxanthellae. The method was previously used to study the photosynthetic pigments of a variety of different classes of marine algae both in unialgal culture (Jeffrey, 1961; Jeffrey and Allen, 1964), and in natural phytoplankton populations (Jeffrey, 1965). However, for resolution and identification of very closely related carotenoids (*e.g.,* isomers of carotene) thin layer techniques using organic or inorganic adsorbents must be used (Chapman, 1966). In the present work the nature of the carotene fraction was established using thin layers of aluminium and magnesium oxide, and the unknown pink xanthophyll (fraction 9) was separated from chlorophyll *c* on thin layers of polyethylene. The sucrose thin-layer method had a resolution similar to the separations obtained with the paper method, except that the separation of each component (particularly the xanthophylls) was more complete, and the method may be used for sensitive quantitative analyses.

The present investigation of photosynthetic pigments in clam and coral zooxanthellae establishes that these symbiotic algae contain the full and characteristic complement of dinoflagellate pigments. On the basis of present knowledge of pigment distribution within algal groups, designation of these zooxanthellae as dinoflagellates is clearly justified.

SUMMARY

1. The photosynthetic pigments of the brown symbiotic algae (zooxanthellae) isolated from five tridacnid clams and nine corals were found to be identical with the pigments of the dinoflagellate *Amphidinium*. Identifications were carried out by two-dimensional paper chromatography and by absorption spectrophotometry. Both zooxanthellae and dinoflagellates contained cholorphylls *a* and *c*, β-carotene, peridinin, neo-peridinin, dinoxanthin, neo-dinoxanthin, diadinoxanthin, and three minor xanthophyll fractions not previously described.

2. Peridinin, crystallized from *Tridacna gigas*, or isolated by paper chromatography from *Tridacna crocea*, *Pocillopora* or the dinoflagellate *Amphidinium*, showed similar absorption characteristics in different solvents. The extinction coefficient of crystalline peridinin in acetone was $E_{1\,\mathrm{cm.}}^{1\%}$ 1340.

3. Clam zooxanthellae (and the dinoflagellates *Amphidinium* and *Gymnodinium*) contained a much higher proportion of chlorophyll *c* than coral zooxanthellae.

4. No chlorophyll decomposition products were found in any freshly prepared zooxanthellae preparations.

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PACEMAKER PROPERTIES OF TUNICATE HEART CELLS

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The primary pacemaker regions are located at the cardiovascular junctions and they alternate in periods of activity which change the direction of peristalsis (see Kriebel, 1968a). It will be demonstrated below that isolated sectors of a cardiovascular junction show about equal pacemaker capabilities and when more than one region is independently active, arrhythmia results. The region which usually functions as the pacemaker was determined and its interaction with latent pacemaker regions is discussed below.

The myocardium is composed of a single layer of musculoendothelial cells, except for a line of undifferentiated cells located opposite the cardio-pericardial raphe; these undifferentiated cells resemble those forming the ring of undifferentiated cells at the ends of the heart (see Millar, 1953). Many investigators have demonstrated that narrow rings of tunicate hearts can beat rhythmically (Bancroft and Esterly, 1903; see Krijgsman, 1956, for a review). However, rings of the heart contain both the raphe and the undifferentiated line of cells, which raises the question whether the myocardial cells themselves have pacemaker properties or whether pacemaker activity is limited to the undifferentiated cells. To investigate this question pacemaker capabilities of pieces of myocardium were examined after both the undifferentiated line and the raphe had been removed.

METHODS

Hearts of adult *Ciona intestinalis* (from Woods Hole, Massachusetts and from California) were used for most experiments. For comparative purposes, hearts of adult *Chelysoma productum, Ascidia callosa* and *Corella willmariana* (from Friday Harbor, Washington) were also used.

Heart contractions in intact animals were observed with the aid of a dissecting microscope and recorded on a kymograph drum. Dissections were performed in sea water at 10° C. *Ciona* heart action potentials were recorded *in situ* by applying suction electrodes to the raphe so that portions of both pericardium and myocardium were sucked into the electrode openings. Electrical activity of the pacemaker regions was recorded by placing as many as four electrodes on the myocardium near the cardiovascular junction. In a second method of recording action potentials, hearts were opened and positioned over a Plexiglass plate containing a row of small electrode openings spaced 1-mm. apart (see Kriebel, 1967a, for details).

Small pieces of heart were spread onto a microscope slide moistened with sea water between two ridges of petroleum jelly. A coverslip holding a drop of sea

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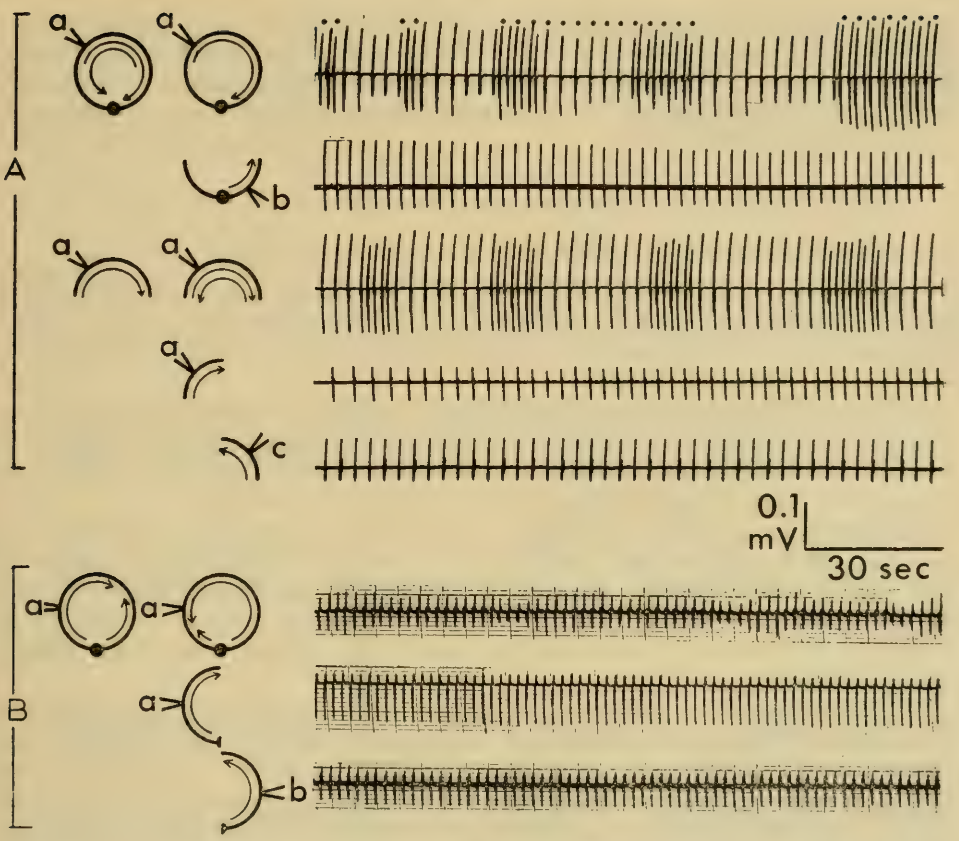

FIGURE 1. Action potentials recorded from isolated pacemaker regions. These hearts were cut in two and collapsed. To the left of each trace the pacemaker region is represented in cross-section either as a circle or, after sectioning longitudinally, as parts of a circle (the raphe is the dot). The electrode positions are alphabetized. The arrows represent directions of conduction which were determined with 3 additional electrodes (not shown). Two arrows passing each other indicate alternating directions of conduction resulting from the activity of two pacemakers (which gives rise to a doubling in beat frequency). Two arrows meeting indicate that the waves of excitation resulting from the activity of two pacemakers cancelled each other. A. Trace 1: Note the periodic doubling in beat frequency. This results from the activity of an ectopic center (action potentials indicated by dots) alternating with the primary pacemaker. The diagrams show directions of conduction during the doubling of beat frequency (left diagram) and when only one center was active. Trace 2: The heart arm has been split in two. Note that this split heart has the same beat frequency as either center in trace 1. Trace 3: Note that the pattern of the doubling in beat frequency was the same as in trace 1. Traces 4 & 5: The myocardium as shown in trace 3 was split. Note that the frequencies of these segments were about the same as that in trace 2 and the frequencies of each center in traces 1 & 3, indicating that all segments of the ring of pacemaker cells had about the same pacemaker capabilities (amplitude was altered by a change in suction). B. Note the periodic, gradual change in signal amplitude in trace 1. A change in signal amplitude resulted from a change in the direction of conduction and the activity of two pacemaker centers. The waves of contraction opposed each other as shown in the diagrams to the left of trace 1. The heart was split in two as shown in the diagrams to the left of traces 2 & 3 and the spread of excitation was unidirectional in each segment.

water was placed over the heart piece, which was pressed until flattened. Sea water (0° C.) was perfused onto one end of the slide and removed at the opposite end by aspiration. With this method of perfusion, pieces of heart tissue continued to contract for several hours. Visual observations of contractions were made with a phase contrast microscope.

Results and Discussion

A. *Patterns of pacemaker activity*

With the exception of conditions to be discussed below, only the end regions of the heart showed pacemaker properties, *i.e.,* contractions always started at one or the other end, or at both ends of the heart; they did not originate anywhere else (see Kriebel, 1968a, for details).

Collapsed hearts *in situ* or in isolation often showed a periodic doubling in beat frequency in one end of the heart. By splitting the heart wall longitudinally, it was possible to separate strips that had the slower rhythm observed in the periods between the times when "doubling" occurred (Figs. 1A and 2A).

In some hearts, generally collapsed, a second active pacemaker could be excited by locally heating a pacemaker region; *i.e.,* in addition to the general increase in frequency, the warming often resulted in a sudden doubling in the number of beats per unit time (see Kriebel, 1968a, for details). This can be readily explained as being due to an ectopic center whose beats occurred between those of the usually active pacemaker.

A second pattern of beating was detected when recording with four electrodes placed around the ostium of an intact but isolated heart, but in contrast to the above pattern of doubling, beat frequency remained the same. In these cases the signal sequence changed, indicating that the direction of conduction had changed and that two pacemakers were active, one on each side of the raphe. The change in the direction of conduction usually changed the signal amplitude (Fig. 1B). When these preparations were split longitudinally, conduction occurred only in one direction and the signal amplitudes remained constant. The alternating dominance between two pacemaker centers in one end of the heart can be compared to pacemaker competition of both ends in the intact heart (Kriebel, 1968a). These results demonstrate that the raphe functionally isolates the pacemaker regions just as it isolates the cells of the general myocardium (Kriebel, 1967a, 1967b).

Arrythmia could be abolished in isolated collapsed hearts by filling them through a cannula and in hearts *in situ* by mechanically stimulating the animal to contract, which raised the blood pressure.

By cutting or crushing parts of the cardiovascular junction containing the pacemaker, it was ascertained that a very small portion, not more than 8% of the circumference of the ostium (6 mm.), was necessary to maintain regular pacemaker activity. During recording from pacemaker regions of collapsed hearts it was often observed that two, sometimes three, sizes of action potentials appeared, each with its own regular frequency. It was particularly revealing that one or more action potentials of a certain series would drop out (Fig. 3C) as if an ectopic center had either failed to reach threshold for active responses or that conduction to the region of recording had failed (conduction is decremental and low at the ends of the heart, which would decrease the safety margin for conduction; Kriebel, 1967a).

Sometimes the electrical records from isolated hearts showed even more complex rhythms. Careful study revealed that the complexities could be resolved into superimposed series of activities of two pacemaker centers, each showing periods of acceleration and deceleration (Figs. 2B and 3A).

B. *Properties of the middle pacemaker* (*the C center of v. Skramlik, 1938*)

Already mentioned previously, under normal circumstances, only the end pacemakers are active. However, when animals with exposed hearts were mechanically stimulated to contract, the blood pressure increased to such an extent that no blood was expelled and the end pacemakers either became irregular or stopped (Kriebel,

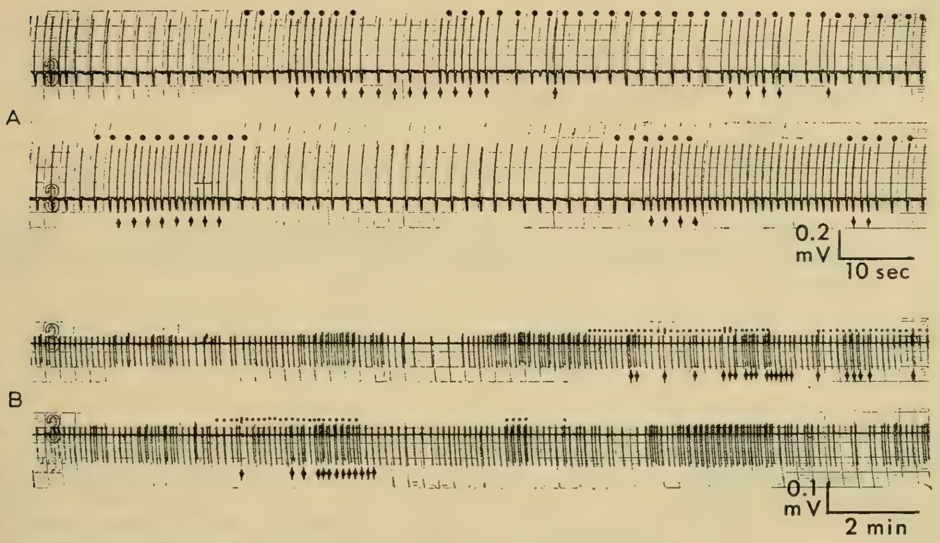

FIGURE 2. Action potentials from *Ciona* pacemaker regions. A. Traces 1 and 2 are continuous. There is little or no slowing in frequency but the frequency either doubled or decreased by half in a rhythmical pattern. Half of the action potentials recorded during the periods of doubling are in phase with those occurring during the preceding or subsequent period of low frequency beating. In trace 1, the dots indicate the action potentials that are in phase, during the doubling in beat frequency, with those preceding doubling. The diamonds indicate the phase of the remaining action potentials. Note that after the period of doubling, the action potentials were out of phase with those preceding the doubling. This activity can be explained by postulating two centers with slightly different frequencies so that a shift in phase would periodically permit both of them to drive the heart. In trace 2, one center was active all the time (dots) and the second center injected extra systoles (some action potentials are indicated by diamonds); 20° C. An opened heart arm. B. Traces 1 and 2 are continuous. In sections of this record, some action potentials in phase are indicated with diamonds or with dots and it is readily seen that the greatest frequencies result from two centers. However, the pacemakers also appear to miss beats as shown in the middle region of the first trace (*cf.* Fig. 3B). During the periods of low beat frequencies, a pacemaker accelerated and decelerated. This is shown just after the diamond symbols in the second trace (*cf.* Fig. 3A). During the periods of doubling, one center occasionally missed beats, presumably because the myocardium was refractory resulting from the prior activity of the dominant pacemaker (these misses are indicated by bars in sequence with the dots); 20° C. Heart arm containing some blood.

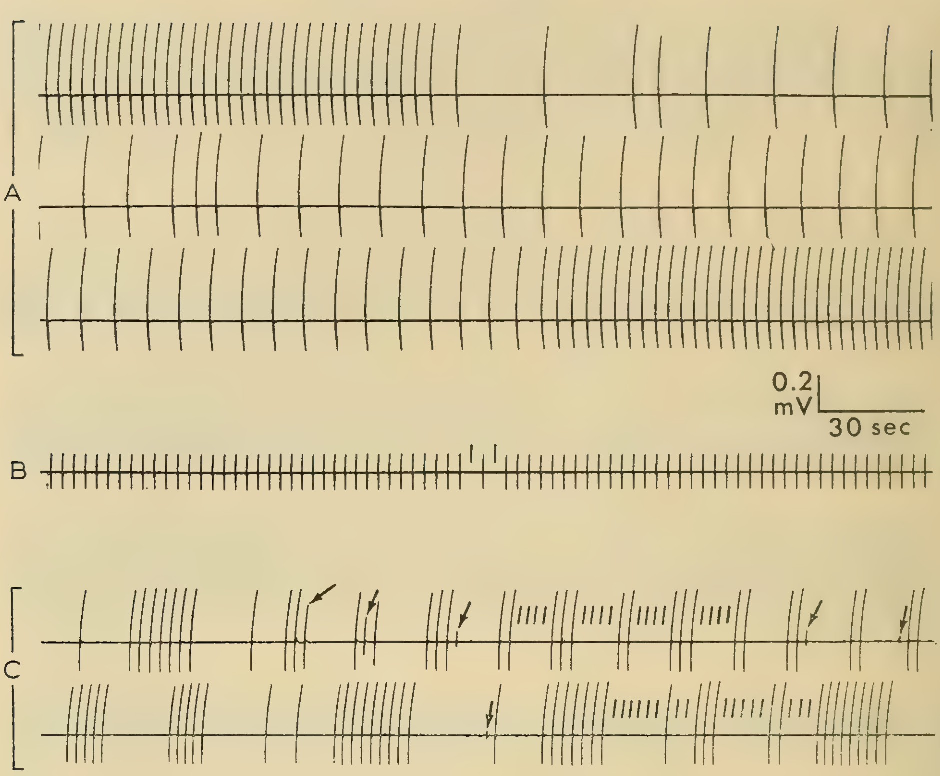

FIGURE 3. Action potentials from isolated pacemaker regions. A. These 3 records are continuous from a half-heart partially filled with blood (electrode in middle of half-heart). In the first trace, the pacemaker decreased in frequency before it stopped. In the second trace and the first half of the third trace the pacemaker gradually accelerated in a manner similar to that *in situ*. However, the beat frequency almost doubled in the middle of the third trace without a gradual increase. This probably resulted from the activity of a second pacemaker center. B. This record is from a collapsed heart arm. In the middle of the record note that two beats were missed (indicated by bars). Note that the action potentials occurring after the misses are in phase with those occurring before the misses. C. Continuous record of action potentials from a collapsed heart arm (electrode near pacemaker region). The intervals between the action potentials of the greatest amplitude are almost constant regardless of the duration of activity. The arrows point to contractions originating from ectopic centers. The bars indicate the missed beats of the pacemaker.

1968a). A few minutes after the end pacemakers had stopped, the C center became active and waves of contraction passed from the middle bend toward both ends of the heart. The frequency was always lower than that of the end pacemakers (in all four species studied).

Localized heating applied to an active C center was not as effective in increasing beat frequency as when applied to an end pacemaker. The Q_{10} of the C center in *Ciona* was found to be about 1.4 and that of the end pacemaker about 2.3 (5–25° C. range; Kriebel, 1968a). It was possible to reverse the direction of contraction by raising the temperature of the end of the heart with the inactive pacemaker or by lowering the temperature of the end with the dominant pacemaker (Kriebel, 1968a). But when both end pacemakers were cooled to about 3° C. (so that their beat frequency dropped to only a few beats/minute) and the temperature of the middle region containing the C center was raised to about 20° C. the C center did not begin activity, although the end-to-end wave of contraction accelerated as it passed along the middle segment of the heart. Therefore a change in tension in the heart wall appears to be the only stimulus in the intact heart which will start the C center. Yet when the middle regions were isolated, the C center began to beat after a few minutes. This suggests that the change in a C center from a dormant to an active pacemaker requires that it not be driven for several minutes. The change from a dormant to an active state in the end pacemakers, after a reversal pause, requires only a few seconds (1–3 beat intervals).

The activity of the C center has been observed by many authors (Bancroft and Esterly, 1903; Hecht, 1918; Benazzi, 1935; Bacq, 1935; Sugi, *et al.,* 1965; Krijgsman, 1956).

C. *Pacemaker properties of myocardial cells*

There are many reports which demonstrate that for many species of tunicates small rings of heart tissue pulsate (see Krijgsman, 1956). However, in rings of heart it has not been determined whether the myocardial cells or the undifferentiated cells are the pacemakers. To answer this question, small pieces of tissue devoid of the raphe and the cells of the undifferentiated line were examind with a phase contrast microscope. They were observed to contract rhythmically, indicating that the cells of the general myocardium have pacemaker properties. Little or no activity was observed initially after isolating a small piece of tissue but after a few minutes 20–50% of the cells contracted at frequencies ranging from a few beats/min. to 12 beats/min. (intact *Ciona* hearts beat at about 20 beats/min.). Adjacent cells could beat independently. A few minutes later, the cells in small areas were contracting together until all the cells of the piece of myocardium contracted synchronously. The transition from contractions of small localized areas to synchronized beating of the entire piece of tissue was usually so rapid that the recruitment of additional fibers was seldom observed.

As in cultured chick heart cells (Smith and Berndt, 1964), there was no morphological difference between beating cells and quiescent cells. Synchronous beating of small groups of cells sometimes persisted for over an hour (also observed by Smith and Berndt, 1964). Occasionally single cells pulsated independently while all neighboring cells contracted together; however, after a period of time the aberrant cells usually followed their neighbors. Independent cell activity can be expected since the safety margin for conduction is very low and the excitability of the tissue is low (Kriebel, 1967a). For example, only local contractions were produced by stimulation with suction electrodes with tip openings less than 20 μ in diameter (Kriebel, 1967a). This means that several cells must be depolarizing simultaneously in order to generate a propagated wave of excitation. In the intact heart, dormant (or latent) pacemaker cells were being driven by the dominant pacemakers. This is substantiated by the fact that no intracellular pacemaker potentials were recorded from the cells of the general myocardium in the intact heart (Kriebel, 1967a, 1967b). The transition from dormant to active states in the cells of the general myocardium requires more time than is required by the end pacemaker (after a pause during reversal) or by the C center. Since one cell cannot drive the heart, the appearance of an ectopic center depends on synchronized activity of several cells, each of which is contributing to the pacemaker current. Assuming that the pacemaker cells at the ends of the heart are functionally similar to the cells of the general myocardium, ectopic centers in the general myocardium are probably similar in size (number of cells) to those at the ends of the heart. Thus, the question arises: what factors integrate the pacemaker cells so that only one center is active in the normally functioning heart? Spread of excitation is by local current flow (Kriebel, 1967a, 1967b, 1968b). At the ends of the heart, the safety margin for conduction is very low because conduction velocity is low and decremental in nature (Kriebel, 1967c). Consequently, any factor that lowers the excitability of the tissue will lower the safety margin for conduction and in turn permit ectopic centers to develop (*cf.* Hoffman, 1965, for vertebrate hearts). Since arrhythmia was more frequent in isolated hearts, it seems reasonable to conclude that the safety margin for conduction was lower in sea water than when the hearts were in blood. However, in collapsed hearts (both *in situ* and isolated), arrhythmia was usually abolished by increasing the blood pressure. This indicates that stretch may increase the safety margin for conduction in the pacemaker region. Either one pacemaker center could drive the other potential pacemaker cells in the end pacemaker region so that they become dormant pacemakers or all of the cells in the end pacemaker region show pacemaker potentials but are driven by one center.

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SUMMARY

1. The primary pacemakers in the tunicate heart are located near the cardiovascular junctions close to the raphe that connects the V-shaped heart to the pericardium. However, small isolated pieces of the ring of myocardial cells at the ends of the heart were found to have nearly equal pacemaker capabilities. Arrhythmia in one primary pacemaker region was found to result from activity of two or more centers.

2. Following isolation of a small piece of tissue in sea water, 20–50% of the cells were observed to pulsate and usually within a few minutes all cells contracted synchronously. Reversals in the direction of conduction in strips of myocardium without any primary pacemaker region were observed.

3. The C center located in the middle of the heart was found to be dormant during normal heart activity but in opened animals it was activated by increasing the blood pressure.

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CHOLINOCEPTIVE AND ADRENOCEPTIVE PROPERTIES OF THE TUNICATE HEART PACEMAKER

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Functionally, tunicate hearts resemble those of the higher chordates since conduction is from cell to cell by local current flow (Kriebel, 1967b, 1968c, 1968d) and the pacemakers are myogenic (Millar, 1953; Kriebel, 1968b). However, cardiovascular control in tunicates has not been extensively studied.

There are no continuously active nerves that maintain the beat frequency either above or below the intrinsic frequency of the pacemaker (something like vagal tone in the higher vertebrates) since ganglion extirpation does not alter the beat frequency (Day, 1921; Bacq, 1935). Stimulation of the ganglion is without effect on the heart rate (Schultze, 1901). However, it is not excluded that there are cardiovascular nerves that are active only during specific conditions.

If regulatory nerves are present, it seems likely that the transmitters would be the same as in the higher chordate. Although acetylcholine and cholinesterase are present in tunicates (Florey, 1963), Ach at low concentrations has no effect on the beat frequency of intact isolated hearts (see Krijgsman, 1956, for a review; Krijgsman and Krijgsman, 1959). Adrenaline has been reported to accelerate the heart beat, but high concentrations were required (10^{-3} g./ml.; Scudder *et al.*, 1963).

Since there are no transverse channels (or gaps) between cells, the myocardium is an effective barrier to substances that do not penetrate cell membranes (Kriebel, 1968d). The results presented here show that Ach and adrenaline do not cross the heart wall and that receptive sites are present only on the lumen surface.

METHODS

Hearts of large adult *Ciona intestinalis* (from California) were exposed by cutting through the test and body wall into the coelomic cavity (see Kriebel, 1968a). Hearts ranged from 24 to 45 mm. in length. In some preparations the blood vessels were ligated before cutting in order to free the heart, whereas in others, the vessels were simply severed. In all preparations the pericardium was opened. Many isolated hearts were ligated in the middle region so that the activity of one primary pacemaker could be studied without the influence of the opposite pacemaker. Hearts were opened so that the lumen surface was exposed to the bath either by cutting along the raphe, or, during electrical recording, by pressing a razor blade through the myocardium. The latter method of opening hearts did not disturb the tissue that was sucked into suction electrodes (Kriebel, 1968a).

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Hearts were carefully secured with small hooks to the bottom of a 5-ml. bath (10° C. or 21° C.) to insure that constant dimensions in the heart wall were maintained during addition of the drug solutions and the washing procedure. Slight alterations in tension changed the amount of tissue sucked into the electrode openings and thereby altered the signal amplitude as well as the excitability of the pacemakers (Kriebel, 1968a). Consequently, control experiments, which consisted of adding, mixing and removing sea water, were performed before and after the drug additions to validate the drug actions. Hearts with pacemakers that initially or later became sensitive to alterations in the bath volume were not used.

The effect of drugs on the beat frequency was tested on hearts, first intact, then opened. A solution of the drug in sea water at a temperature equal to that of the bath was added with a pipette. The final concentrations were predetermined. Mixing was accomplished within 15 seconds with an eye dropper. To remove a drug solution at least 100 ml. of sea water of the desired temperature were perfused through the bath.

Acetylcholine chloride (Merck), L epinephrine bitartrate (Nutritional Biochemical Corporation), d-tubocurarine chloride pentahydrate (Burroughs Wellcome & Co.) and atropine sulphate (The Norwich Pharmacal Co.) were used.

RESULTS

Since the effects of drugs on the pacemaker frequencies of intact and opened hearts were to be compared, and since the amount of intracardiac blood affects the beat frequency (Kriebel, 1968a), intact hearts were studied in the collapsed condition (*i.e.*, they contained no blood). Thus, when the hearts were opened, the pacemakers were not subjected to a change in tension. However, arrhythmia frequently occurred in collapsed hearts (Kriebel, 1968a, 1968b). A common pattern of arrhythmia was a doubling in the beat frequency due to the activity of an ectopic center (as shown in Fig. 1A, trace 1).

Acetylcholine (10^{-8} to 10^{-4} g./ml.) and adrenaline (10^{-6} to 5×10^{-4} g./ml.) were without effect on the beat frequency of intact isolated *Ciona* hearts (and half-hearts; with and without the blood vessels tied off, full of blood or collapsed). However, when the hearts were opened, forming a flat sheet of tissue, Ach at 10^{-8} to 10^{-7} g./ml. or adrenaline at 10^{-5} g./ml. initially stopped the primary pacemaker (Figs. 1 and 2). The pacemakers did not again stop when the bath was stirred or when another threshold aliquot of drug was added. The concentration could be slowly raised to about 100 times threshold for Ach and about 10 times for adrenaline with no effect on the beat frequency of the primary pacemaker. Therefore, the response of the pacemakers to these agents exhibited tachyphylaxis, *i.e.*, they became desensitized. However, at initial concentrations greater than 100 × threshold for Ach and 10 × threshold for adrenaline the primary pacemakers were irregular and ectopic centers developed (Ach at 10^{-5} g./ml., Fig. 2; and adrenaline at 5×10^{-4} g./ml., Fig. 1).

Even when vessels were not ligated; intact isolated hearts were insensitive to the drugs, presumably because the blood vessels sealed when they were severed. When the vessels were opened and washed, intact hearts became sensitive to Ach and adrenaline. However, the concentrations required to arrest the hearts were

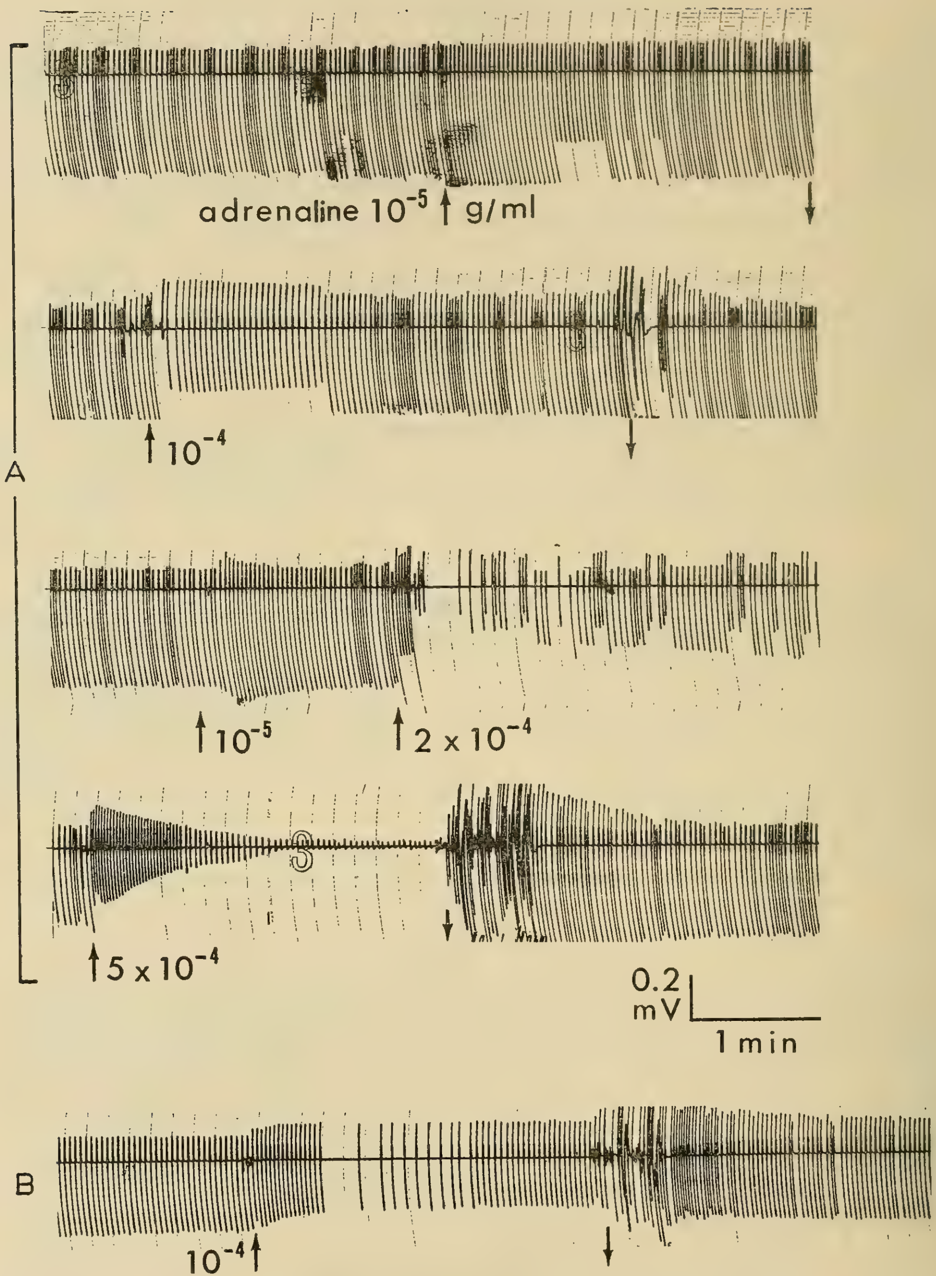

FIGURE 1.

higher than required after the hearts were opened. This indicates that diffusion through the collapsed ostia is slow and in addition it is likely that tachyphylaxis occurred.

Atropine antagonized the effect of Ach (Fig. 2). Threshold concentrations of d-tubocurarine (10^{-7} to 3×10^{-7} g./ml.) either slowed or stopped a pacemaker center. Complete stoppage of the heart occurred with slightly greater concentrations and primary pacemakers did not recover. The effect was additive to that of Ach.

Adrenaline sometimes stopped the heart for a few beats permitting an ectopic center to develop which alternated with the primary pacemaker center, doubling the beat frequency (Fig. 1A, trace 4; *cf.* Scudder et al., 1963; Sugi and Matsunami, 1966). Doubled beat frequency also occurs frequently in deflated hearts and can be produced by locally heating the pacemaker region (Kriebel, 1968a, 1968b). If a primary pacemaker and an ectopic center were active in a heart arm, a threshold concentration of adrenaline (10^{-5} g./ml.) often stopped one center, resulting in halved beat frequency (Fig. 1A, trace 1).

High concentrations of adrenaline (5×10^{-4} g./ml.) decreased conduction velocity (Kriebel, 1967a). This can explain the decrease in signal amplitude (also found by Scudder *et al.,* 1963). After addition of a high concentration of adrenaline, conduction velocity continued to decrease for 1 to 3 minutes. During this time, amplitude of action potentials also gradually decreased (Fig. 1A, trace 4). Although beat frequency decreased under the influence of adrenaline, groups of beats at a relatively high frequency occurred. By the shape of the recorded wave form they could be attributed to one pacemaker and it appears that beats were missed between the groups (as seen in Fig. 1A, trace 3). Because conduction velocity is very low and because it is decremental at the ends of the heart (Kriebel, 1967a) a further decrease in the conduction velocity due to the action of adrenaline would decrease the safety margin for conduction. A reduced safety margin could give rise to missed beats and permit the development of ectopic centers.

FIGURE 1. Effect of adrenaline on the beat frequency of opened *Ciona* hearts. The electrograms were recorded with suction electrodes placed on the primary pacemaker region. The downward arrows indicate washing; 20° C. A. Trace 1: The first half of this record shows a rhythmical doubling in beat frequency attributed to the activity of an ectopic center which alternated with the activity of the primary pacemaker. After the application of adrenaline note that the doubling in beat frequency was stopped for over 1 minute. Trace 2: After the application of adrenaline at a concentration of 10^{-4} g./ml. the heart stopped, the doubling in frequency was abolished and for about 2 minutes the heart contracted at a lower frequency. However, it was visually determined that the direction of contraction had reversed. That is, an ectopic center at the cut end of the heart arm now drove the heart. The activity of the ectopic center is indicated by the change in the signal amplitude. Trace 3: With adrenaline 10^{-5} g./ml. the doubling in beat frequency was stopped as in trace 1. With adrenaline 2×10^{-4} g./ml. the heart did not recover. Signals of different amplitude indicate a change in the direction of conduction and thus the activity of different centers. Note that the signals of the same amplitude sometimes occurred in groups (see text). Trace 4: Continuous with trace 3. Adrenaline at 5×10^{-4} g./ml. doubled the beat frequency for about 40 seconds. Then the beat frequency suddenly dropped to about half, at which time it was visually observed that the wave of contraction started at the cut end of the half-heart. B. This electrogram shows the typical effect of adrenaline on an active pacemaker. The slight increase in frequency just after drug application was probably due to an increase in the bath temperature. Mixing was 30 sec. after arrow.

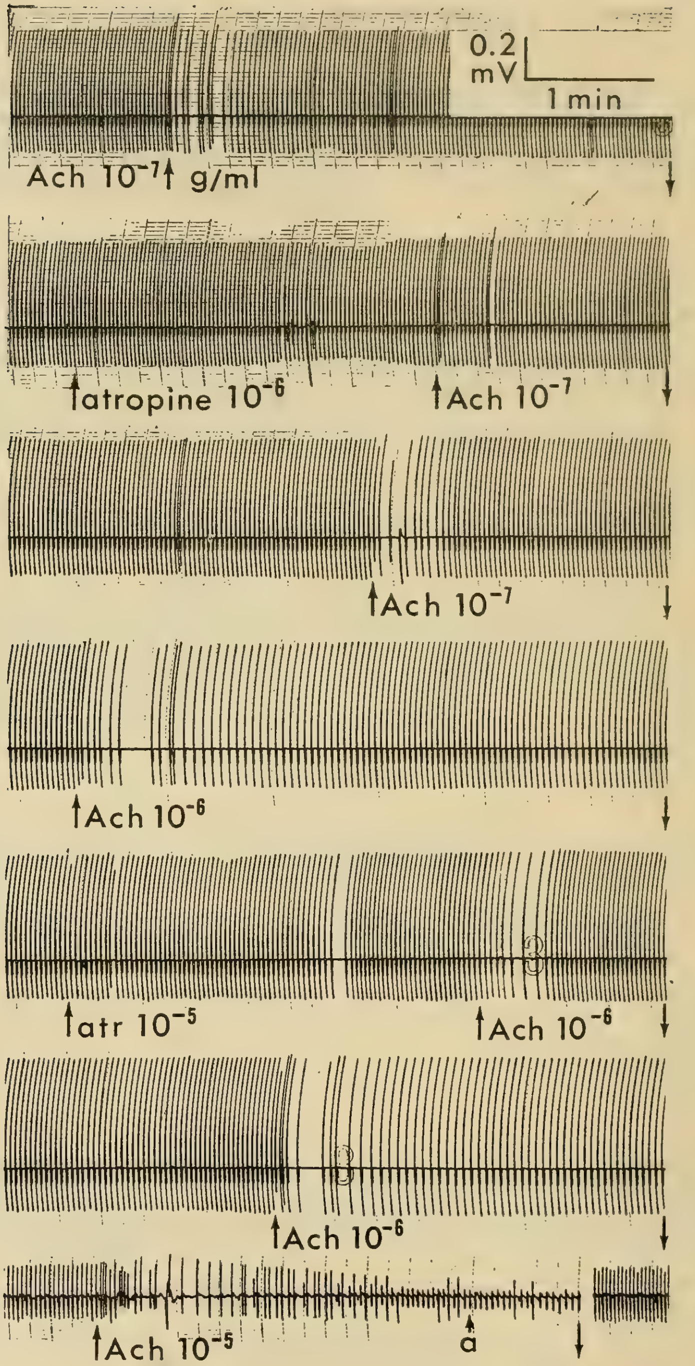
0.2 mV
1 min
Ach 10^{-7} g/ml
atropine 10^{-6}
Ach 10^{-7}
Ach 10^{-7}
Ach 10^{-6}
atr 10^{-5}
Ach 10^{-6}
Ach 10^{-6}
Ach 10^{-5}
a

FIGURE 2.

DISCUSSION

Tight junctions join all cells together at their apical borders and exclude extracellular space so that there are no transepithelial diffusion channels between cells (Kriebel, 1968d). The ineffectiveness of drugs on intact hearts shows that the heart tube is permselective and that cholinoceptive and adrenoceptive sites are on the lumen surface.

The failures of other investigators to observe the effects of low concentrations of Ach and adrenaline are readily explained by the insensitivity of the unopened heart, the sealing of the cut ends of the heart and by the presence of tachyphylaxis (*cf.* Bacq, 1934; Krijgsman and Krijgsman, 1959; Scudder *et al.,* 1963; Sugi and Matsunami, 1966).

The absence of an accelerating effect of adrenaline, as found in other chordate hearts, may be an indication of the absence of cardioacceleratory nerves.

On the other hand, the typical cholinoceptive properties of the myocardium (inhibition caused by Ach and Ach-block by atropine) favors the interpretation that the tunicate heart may receive cardioinhibitory innervation (Florey, 1951) and this innervation is cholinergic. In this connection it is interesting that Bone and Whitear (1958) could trace nerves as far as the pericardium.

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SUMMARY

1. Many previous investigators have reported that acetylcholine has little or no effect on the beat frequency of intact but isolated hearts. However, it was found that when hearts were split open, Ach at low concentrations (10^{-8} g./ml.) stopped the heart beat for up to a minute. Atropine blocked Ach.

2. D-tubocurarine at a concentration of 3×10^{-7} g./ml. stopped the beat of opened hearts. Its effect was additive to that of Ach.

3. Adrenaline (10^{-5} g./ml.) could stop pacemakers. However, a doubling in beat frequency was frequently observed with higher concentrations. Doubling in frequency resulted from an ectopic center which alternated with the primary center, thus driving the heart at twice its original frequency. In hearts which showed arrhythmia resulting from activity of more than one pacemaker center,

FIGURE 2. Effect of acetylcholine on the beat frequency of an opened *Ciona* heart. The electrograms were recorded with a suction electrode placed on the myocardium near the end of the heart (*i.e.,* on the primary pacemaker region). The downward arrows indicate washing. Temperature 20° C. Traces 1–3 show that atropine blocked a threshold concentration of Ach. Traces 4–6 show that atropine partially blocked the initial stoppage caused by a high concentration of Ach (10^{-6} g./ml.) and greatly diminished the time required to regain the original beat frequency. Trace 7 is from the same heart but the electrode position had been slightly changed altering the signal amplitude. Note the different amplitude after application of Ach. It was visually observed that the smallest signals (a) corresponded to contractions starting at the cut end of the half-heart.

adrenaline at a low concentration (10^{-5} g./ml.) usually stopped one or the other of the centers, decreasing the beat frequency by half.

4. Hearts recovered in low concentrations of either Ach or adrenaline. However, at higher concentrations (Ach 10^{-6}, adrenaline 5×10^{-4} g./ml.) arrhythmia was induced and hearts did not recover. The occurrence of arrhythmia is discussed with respect to the decremental nature of conduction in the ends of the heart and to the observation that all cells near the ostia are latent or dormant pacemakers.

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INTER–PHYLOGENETIC SPECIFICITY IN THE BONDING OF AMINO ACIDS TO tRNA[1]

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The first step in protein synthesis is generally considered to be the reaction of a free amino acid (AA) with adenosine triphosphate (ATP) and an appropriate "activating" enzyme to form an "activated" amino acid (probably 5′-aminoacyl adenylate; AA ~ AMP) and pyrophosphate (Hoagland, Zamecnik and Stephenson, 1957).

This activated amino acid reacts with the 2′ or 3′ OH of a low molecular weight ribonucleic acid (tRNA) to release AMP and produce an aminoacyl tRNA which subsequently transports the amino acid into the appropriate part of the protein being synthesized. Evidence for this unique RNA was adduced more or less simultaneously and independently by Holley (1957) and by Hoagland *et al.* (1957), as well as indirectly by Hultin (1956) and Hultin and Beskow (1956). The structures of several tRNA's have been determined and it seems quite likely that more than one tRNA accepts a particular amino acid, although there is only limited evidence that more than one aminoacyl tRNA ligase exists for each amino acid (Yu, 1966; Barnett, Epler and Brown, 1967).

It is presumed that some sort of base pairing of the aminoacylated tRNA with genetic nucleic acid (mRNA, virus RNA or even DNA) must account for the extraordinary precision with which amino acids are assembled into proteins (Loftfield, 1963) or with which phenotypes reflect different genotypes. There is some evidence that the genetic code is universal and corresponding evidence that the insertion of an amino acid into a particular locus depends only on the tRNA to which it is attached. Berg and Ofengand (1958) showed that the activating enzymes also catalyzed the transfer of amino acid to tRNA. Hecht, Stephenson and Zamecnik (1959) showed that most, if not all, tRNA's terminated in an adenylic-cytidylic-cytidylic trinucleotide and several recent studies on structure show that the secondary structures may be similar for all tRNA's. It was the purpose of this work to establish whether activating enzymes from widely different sources could aminoacylate tRNA's from other phyla. Although the results are not gratifyingly unequivocal, we find that interaction between heterologous pairs exists where others have found it absent. In some cases, the interaction of heterologous pairs of tRNA and enzyme, as measured by our methods, is better than between homologous pairs. Our techniques show that the aminoacyl tRNA is chemically active however obtained, and that there is no chemical evidence of a multiplicity of aminoacyl tRNA's.

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[1] Some of this material has been presented at the Tuesday Evening Seminar Series at the Marine Biological Laboratory (Loftfield and Eigner, 1967a). This work has been supported by U.S.P.H.S. Grant CA 08000.

MATERIALS AND METHODS

(A) L-1-[^{14}C]-valine and L-1-[^{14}C]-leucine (20 c.p.m. per $\mu\mu$mole) were prepared by the Bucherer hydantoin synthesis (Loftfield and Eigner, 1966). TRIS (tris (hydroxymethyl)-aminomethane), ATP (adenosine triphosphate), and other reagents were purchased from commercial sources.

(B) Preparation of enzymes—*E. coli.* Ten g. of *E. coli* strain B cells were suspended in 50 ml. of .02 *M* TRIS buffer pH 7.5 containing 600 mg. of reduced glutathione per liter. The cells were ruptured by high pressure passage through the orifice of a Ribi cell fractionator. The suspension was then centrifuged two hours at 27,000 *g*. The supernatant fluid possessed a high concentration of both valine and leucine tRNA ligases and was generally used as such. Occasionally, the enzymes were further purified by column chromatography on DEAE cellulose (Berg, Bergmann, Ofengand and Dieckmann, 1961).

Toadfish. One gram of toadfish liver was homogenized in a Potter-Elvehjem homogenizer with 1 ml. of Medium A (0.35 *M* sucrose, 35 m*M* $KHCO_3$, 4 m*M* $MgCl_2$ and 25 m*M* KCl). The suspension was centrifuged at 24,000 *g* for 30 minutes. The supernatant phase was removed and the sediment extracted with a second 1-ml. portion of Medium A. All of the valine and leucine activating enzymes appeared in the supernatant phases which were used as such.

Starfish. The gonads, male or female, appeared to be the best source of amino acid activating enzymes. Ripe gonads were minced and stirred gently one-half hour in fresh sea water to release eggs or sperm. The water with eggs or sperm was decanted and the suspension of gonad tissue was homogenized in a Potter-Elvehjem apparatus with an equal volume of Medium A containing 600 mg./l. of reduced glutathione. The suspension was centrifuged 10 minutes at 20,000 *g* and the supernatant phase was used as the enzyme source.

Yeast. Fresh bakers yeast was cultured and harvested in log phase by Dr. J. Scaletti of The University of New Mexico. Fifty ml. of packed cells were suspended in 100 ml. of 0.02 *M* pH 7 phosphate buffer containing 600 mg./l. of reduced glutathione and forced through a Ribi press at 35,000 lbs./in.^{2} The suspension was centrifuged 20 minutes at 20,000 *g* and $(NH_4)_2SO_4$ was added to 80% saturation. After 20 minutes at 4° C., the precipitated protein was collected by centrifugation at 20,000 *g* for 20 minutes. The precipitate was dissolved in 5 ml. of the above buffer, 1 g. of Bentonite was added and the slurry was allowed to stand at room temperature 30 minutes. The Bentonite was removed by centrifugation at 20,000 *g* and the enzyme solution was dialyzed against the phosphate glutathione buffer for six hours at 4°. The RNA contamination could be reduced by treating each ml. of the enzyme solution with 10 μmoles of $MgCl_2$, 600 μg. of glutathione and 10 mg. of streptomycin for 30 minutes at 0°. The precipitate was removed by centrifugation and the solution again dialyzed against the phosphate glutathione buffer.

Each enzyme preparation was routinely examined for activity using the [^{14}C]-amino acid hydroxamate technique (Loftfield and Eigner, 1963).

(C) tRNA's.—*E. coli* tRNA and yeast tRNA were gifts from Schwartz Bioresearch.

Starfish.—Eighty ml. of ripe starfish eggs were suspended in 80 ml. of 0.001 *M* pH 7.5 TRIS buffer together with 16 ml. of 0.1 *M* $MgCl_2$. The suspension was homogenized at 0° for 5 minutes in a Waring Blendor. Undissolved material was removed by centrifugation for 10 minutes at 16,000 *g*. The supernatant phase was gently mixed for one hour at room temperature with an equal volume of 90% phenol. Centrifugation separated the mixture into two phases. To the upper phase were added 0.1 volume of 20% potassium acetate and 2.5 volumes of ethanol. After 16 hours at −10°, the precipitate was collected by centrifugation, washed once with 67% ethanol and drained. The precipitate was then suspended in 1 *M* NaCl and centrifuged at 15,000 *g* for 30 minutes to sediment most of the ribosomal RNA. The supernatant was dialyzed against water, treated with 0.5 *M* TRIS at pH 8.8 for 45 minutes to hydrolyze aminoacyl tRNA, neutralized with acetic acid and dialyzed again. Although the solution showed a spectrum typical of RNA, it was very viscous, suggesting the presence of a highly polymerized contaminant. Therefore the solution was stirred two minutes at 20° with one volume of 2.5 *M*, pH 7.5 potassium phosphate buffer and one volume of methyl cellosolve. The mixture was separated into two phases by centrifugation at 15,000 *g* for 10 minutes at 4°. tRNA was precipitated from upper phase by adding potassium acetate and alcohol as above.

Toadfish.—Forty grams of toadfish liver were homogenized and worked up in much the same way as the starfish eggs. Although the product had a slight gray-brown color, it had an ultraviolet spectrum that was typical of tRNA. Like each of the other tRNA's prepared, the ratio of $\mathrm{O.D.}_{260}/\mathrm{O.D.}_{280}$ was approximately 2.0.

Using homologous enzymes, the maximum aminoacylation of each tRNA was: in mμ moles per mg.; *E. coli,* leucine 1.0, valine 1.2; yeast, leucine 0.37, valine 0.35; starfish, leucine 0.45, valine 0.45; toadfish, leucine 0.59, valine 0.36.

(D) The aminoacylation of tRNA was carried out as previously described (Loftfield and Eigner, 1967a) and as summarized in the lengend of Figure 1.

RESULTS

There has been an abundance of work on the cross-reaction of activating enzymes and tRNA's of different species. For the most part only bacterial, yeast and vertebrate sources have been used and examination has been confined to a few amino acids. In general, there seems to have been some cross-reaction when yeast or vertebrate sources of enzyme and RNA were used but none or little when bacteria provided one of the ingredients. Benzer and Weisblum (1961) showed that the extent of cross-reaction varied with the amino acid used. We (Loftfield and Eigner, 1963) demonstrated that if sufficient care is taken, pairs reported to have no cross-reaction can be shown to react, though the rates may be less than 1% of the homologous pairs.

Stulberg and Novelli (1962; p. 422) correctly point out some of the errors in making hasty statements that interspecies interaction does not obtain. Frequently so-called "tRNA" has not been shown to accept amino acids from the homologous enzyme. Thus many of our preparations of starfish "tRNA" had the physical and chemical properties of tRNA without being able to bind covalently significant amounts of several amino acids even using starfish enzyme. Other workers have occasionally reported no effort to determine whether the aminoacylated tRNA was biochemically or chemically active.

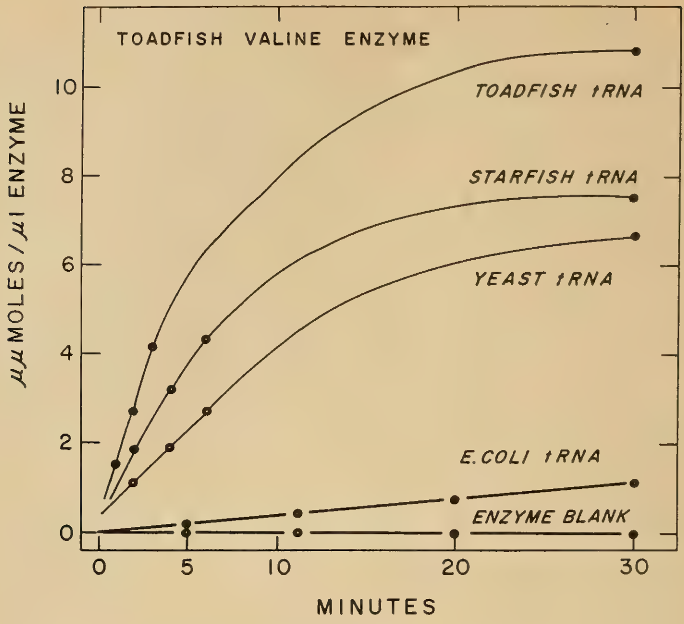

FIGURE 1. A typical experiment that shows the rate of valyl tRNA formation by the toadfish valine activating enzyme with four different species of tRNA. The reactions were carried out at 25° with 10 m*M* ATP, 0.1 *M* TRIS (pH 7.5), 30 μ*M* [^{14}C]-valine, 0.5 mg. tRNA per cc., and 6 μl. enzyme solution per tube (final vol. per tube 225 μl.; four aliquots of 50 μl. each were taken at times indicated). The enzyme blank contains everything except added tRNA. The aliquots were worked up as previously described (Loftfield and Eigner, 1967a). The 30-minute observations correspond to the incorporation of 0.35 mμ mole of valine to 1 mg. of yeast tRNA and to 0.45 mμ mole of valine into 1 mg. of starfish tRNA, exactly the same saturating values as found with homologous enzyme. On the other hand, the *E. coli* tRNA is only about one-tenth saturated.

Hence the current work has been undertaken with considerable care to establish that aminoacylation of tRNA was actually taking place. Every figure noted in Table I represents a rate of reaction drawn from at least two different experiments in which the extent of reaction had been determined at no fewer than three time intervals. Figure 1 illustrates the kind of data used and the difficulties involved in comparing rates. For instance, many of our enzyme preparations contained an RNase which slowly destroyed the substrate RNA; this was clearly a greater problem if the reaction was slow or if larger amounts of enzyme solution were being used. Thus in some heterologous reactions similar to Figure 1, the tRNA was never saturated with amino acid. However, the addition of ATP and enzyme homologous with the tRNA at 30 minutes was ineffective. The receptor competence of the tRNA had been destroyed during the incubation. This was especially evident when enzyme and tRNA were pre-incubated without the amino acid. Depending on the species and preparation, the competence of the tRNA to accept amino acid steadily deteriorated. No two preparations of tRNA accepted, even from homologous sources, the same amount of leucine or valine and we (Loftfield and Eigner, 1963, 1965) have previously shown that either aminoacylated tRNA or damaged (oxidized) tRNA inhibits the reaction. Even products of tRNA digestion have been shown to be inhibitory (Hayashi and Miura, 1966; Letendre, Michelson and Grunberg-Manago, 1966). We have presented in Table I relative initial rates of reaction where destruction of substrate, product inhibition and enzyme inactivation are minimized.

With due regard for all these difficulties, Table I contains material of interest. In keeping with earlier observations, most *E. coli* enzymes of established activity interact poorly if at all with non-bacterial tRNA's of established competence while there is a fair to better than natural interspecies reaction when the enzymes and tRNA's are derived from yeast or the higher organisms. (At best, this is only a weak generalization; starfish valine enzyme reacts poorly if at all with *E. coli* tRNA while the leucine enzyme of starfish is as good as the *E. coli* enzyme.)

TABLE I

Relative rates of homologous and heterologous enzymic formation of aminoacyl tRNA's. The rates are expressed as per cent of the rate obtained for homologous tRNA and enzyme under standard conditions

Enzyme		*tRNA*			
		E. coli	Toadfish	Starfish	Yeast
E. coli	val	100	2	1.5	0.5
	leu	100	<0.1	<0.5	2
Toadfish	val	3	100	50	27
	leu	<0.1	100	47	3
Starfish	val	<1.	100	100	83
	leu	100	40	100	40
Yeast	val	40	200	140	100
	leu	45	300	65	100

Most striking is the observation that yeast enzymes specific for both valine and leucine are more active towards starfish and toadfish tRNA than they are towards yeast tRNA. Equally notably, yeast enzymes for both amino acids are quite active in transferring leucine or valine to *E. coli* tRNA while *E. coli* enzymes are almost inert towards yeast tRNA. Partly to the contrary, starfish enzyme is very active towards *E. coli* tRNA only with leucine while *E. coli* enzyme is poor in transferring either leucine or valine to starfish tRNA.

There is a concern whether some tRNA normally specific for one amino acid is being aminoacylated by another. Although there are several reports of multiple tRNA's specific for a single amino acid there are to date, only a few suggestions of a multiplicity of the aminoacyl tRNA ligases (Yu, 1966; Barnett *et al.*, 1967). Nonetheless we deliberately used crude enzyme preparations rather than purifying the enzyme so as to lose no active component. If our enzymes were transferring amino acids indiscriminately to a variety of the heterologous tRNA's, we might expect that some tRNA's would accept more leucine or valine than they did from the homologous enzyme. In every case, we found that the heterologous aminoacylation yielded only the same extent of reaction as the homologous. (Note the legend in Figure 1.) No evidence was found to indicate that the heterologous reaction was less specific than the homologous reaction.

The most valid criterion of whether a heterologously aminoacylated tRNA was identical with the same tRNA aminoacylated with a homologous enzyme would be to compare the behavior of the two tRNA's in the synthesis of a specific protein. Unfortunately all cell-free syntheses of protein are suspect. The use of either natural messenger RNA (*i.e.*, to synthesize hemoglobin or β-galactosidase) or so-called synthetic mRNA's like poly-uridylic acid (to synthesize polyphenylalanine) leads to data in which high backgrounds, autolysis, RNase activity, salt

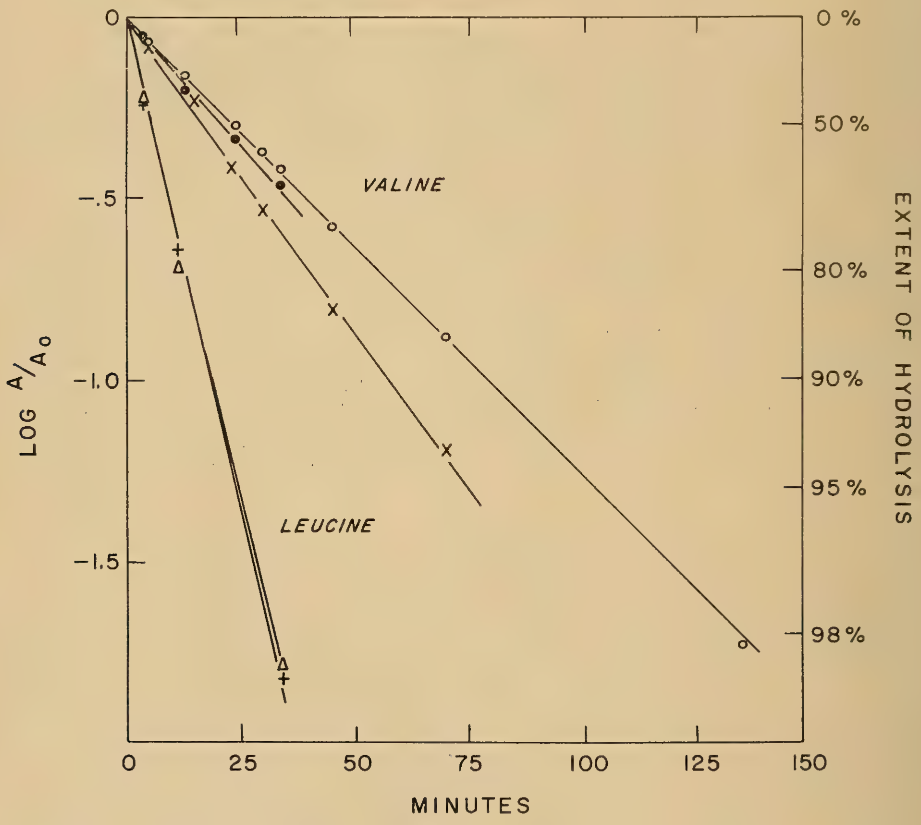

FIGURE 2. Rate of stripping of [^{14}C]-valyl and [^{14}C]-leucyl tRNA. The tRNA was labeled completely in each case at 25°, then made 0.16 *M* in Na_2CO_3 (pH 10.3) and brought to 35° to initiate the stripping reaction. Other conditions same as Figure 1, but larger amounts of enzyme were used. [^{14}C]-leucine was used with *E. coli* enzyme and *E. coli* tRNA (△), and toadfish enzyme and toadfish tRNA (+). [^{14}C]-Valine was reacted with *E. coli* enzyme and *E. coli* tRNA (○), toadfish enzyme and toadfish tRNA (×), and toadfish enzyme and starfish tRNA (●).

concentrations, etc., are prominent contributors to an ambiguous interpretation. Accordingly, we have used a much simpler criterion of the nature of the aminoacyl tRNA bond. Raacke (1958) pointed out that, although aminoacyl tRNA compounds are essentially simple esters, they are far more reactive than the aliphatic esters of amino acids. We chose to measure the rate of deaminoacylation of the aminoacyl tRNA's formed by either homologous or heterologous enzymic action. Figure 2 is illustrative of the kind of data obtained at pH 10.3 for a variety of these hydrolyses.

The first thing to be noted in Figure 2 is that the first order rate of hydrolysis is absolutely constant, *i.e.*, the semi-logarithmic plot shows no deviation from linearity. In every case, the hydrolysis was followed as far as the available [^{14}C]-aminoacyl tRNA permitted. Thus in the case of *E. coli* valyl tRNA the rate of hydrolysis remains constant until 97% complete hydrolysis. In no case is there evidence of a change in rate which certainly would have resulted from differences in chemical activities of the several leucyl tRNA's or valyl tRNA's. The graph shows clearly that the technique is sensitive to differences in rate of as little as 10%. Raacke showed that amino acids esterified to positions other than the unique 2′ or 3′ adenosyl terminal positions of tRNA would be a hundred times less reactive. Correspondingly, our data show that no more than 3% of the valine or leucine is bound to a relatively unreactive position such as a 5′ hydroxyl or one of the amino or enol residues. Whether the tRNA has been aminoacylated by homologous or heterologous enzyme, the rates of hydrolysis are well within the range established for a particular amino acid. It is noteworthy that the leucyl derivatives are some four times more labile than the valyl derivatives. This agrees with the well recognized differences in rates of hydrolysis of leucyl and valyl peptides and is consistent with the steric inhibition generalizations of Newman (1950). In fact, the relative rates of hydrolysis of the leucyl esters are somewhat less than would have been anticipated from the application of Newman's "Rule of Six" to the straightforward attack of a hdyroxyl ion or other nucleophile on the carbonyl bond.

Particularly to be noted are the clearly discernible differences in rates of hydrolysis of the several valyl tRNA's. Although these are much more stable than the leucyl tRNA's, the analysis is sensitive enough to detect differences which presumably result from differences in the structure of the tRNA.

The extraordinary activity of the yeast enzyme towards toadfish tRNA warranted a closer examination. It is quite conceivable that the high relative rates of aminoacylation are a consequence of our failure to saturate the homologous enzyme tRNA pairs. For instance if the K_m for binding toadfish tRNA to yeast enzyme is very low while the K_m for binding the yeast tRNA to yeast enzyme is high, we might expect that we should observe nearly maximal aminoacylation rates with toadfish tRNA while we were still observing only a fraction of the V_{max} with yeast tRNA. Figure 3 shows that this interpretation is quite incorrect. An Eadie plot of rate data collected at various concentrations of tRNA shows that the K_m for yeast valine tRNA on yeast enzyme is 2.2×10^{-7} *M* while the K_m for toadfish tRNA is 1.9×10^{-6} *M*. There is probably a ten-fold poorer binding of the toadfish tRNA which is more than compensated for by a six-fold increase in the reactivity of the toadfish tRNA as measured by V_{max}. If the observations of

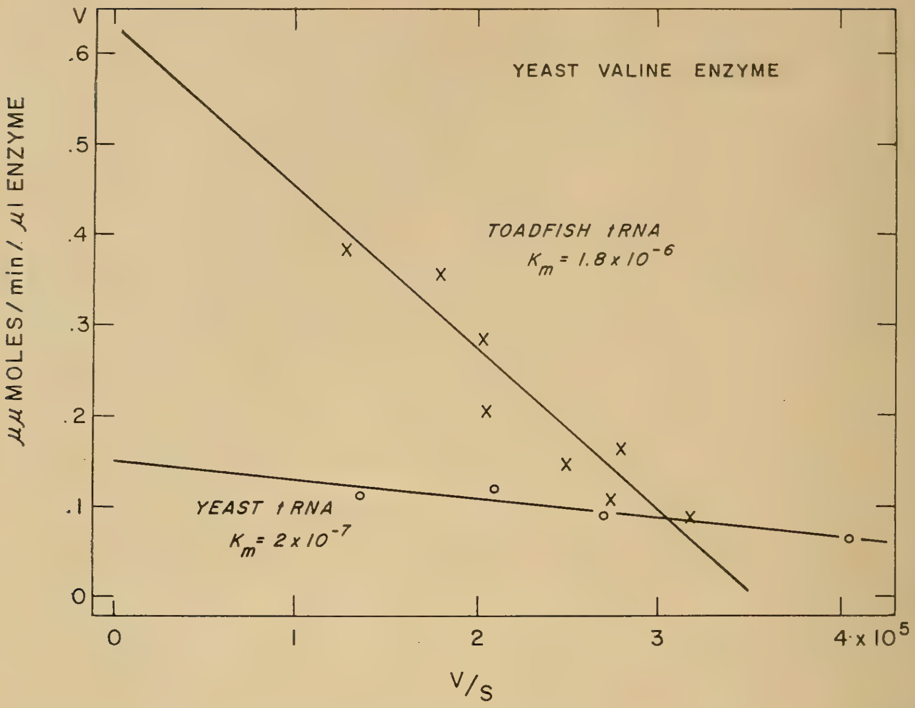

FIGURE 3. Eadie plot of formation of [^{14}C]-valyl tRNA by the yeast valine enzyme. The graph shows that yeast tRNA (○) has a K_m of 2×10^{-7} *M* and a V_{max} of 0.16 μμmoles/min./μl. enzyme. Toadfish tRNA (×) has a K_m of 1.8×10^{-6} but a V_{max} of 0.63 μμmoles/min./μl., four times greater than the V_{max} of yeast tRNA with yeast enzyme. Conditions as in Figure 1, except yeast tRNA varied from 0.4 to 2.0 mg./cc. and toadfish tRNA varied from 0.46 to 4.9 mg./cc.

Table I had been made with tRNA concentrations of .5 mg. per ml., the toadfish tRNA would have appeared to be three or four times more active than yeast tRNA.

DISCUSSION

It has been noted by ourselves and others that there is inter-species interaction of tRNA and the corresponding ligase. It has previously been thought that most non-bacterial tRNA's would interact with other non-bacterial enzymes while bacterial tRNA would not react with non-bacterial enzymes. We (Loftfield and Eigner, 1963) have previously shown some cross reaction between *E. coli* tRNA or activating enzymes and the tRNA or enzymes derived from yeast and ascites tumors even when the rates of aminoacylation were extremely low. The present observations extend the generalization to enzymes and tRNA's from four widely differing sources. As noted previously, the failure to observe interaction as measured by an immeasurably low reaction rate does not mean that there is no interaction. Binding of the heterologous tRNA to enzyme may occur, but may be in an unproductive mode or the rate of reaction may be so low as to escape observation in the presence of adventitious ribonucleases.

Table I shows that either valine or leucine reacts measurably for every pair of enzymes and tRNA's examined. There is no obvious pattern as to which amino acid is more likely to cross-react. For instance, *E. coli* enzyme puts more valine than leucine onto toadfish and starfish tRNA but more leucine onto yeast tRNA.

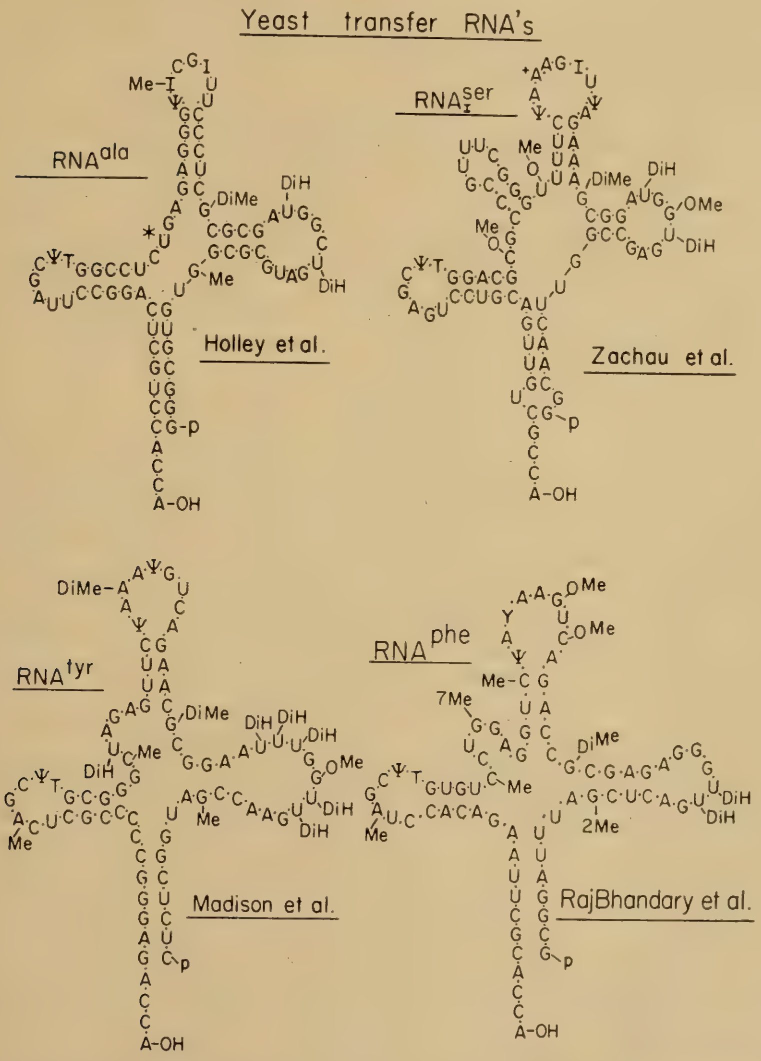
Yeast transfer RNA's
RNA^ala
Holley et al.
RNA^ser_I
Zachau et al.
RNA^tyr
Madison et al.
RNA^phe
RajBhandary et al.
A-OH
A-OH
A-OH
A-OH

FIGURE 4. Possible conformations of four of the five tRNA's of known sequence. The sequences at the bottom (—OH end) and on the left loop are identical or very similar. The other portions, especially the right loop and the upper loop (which contains the presumptive anti-codon), are very unlike and may be responsible for the interphylogenetic amino acid specificity of the tRNA-ligase interaction.

Previously we have shown a similar irregularity in the actual K_m's for the binding of yeast or *E. coli* tRNA's to valine activating enzymes. In the present study this observation too is repeated and extended. Toadfish valine tRNA is bound to yeast enzyme ten times more weakly than yeast tRNA but is apparently many times more reactive once on the enzyme.

Beyond this, we have made no observations that would suggest any heterogeneity in the formed aminoacyl tRNA. In no case did the heterologous reaction proceed further than the homologous and in every case the rate of hydrolysis of the product followed first order kinetics for a homogeneous material as far as the reaction could be followed.

All these observations are consistent with the view that all valine specific tRNA's share some features in common which are not present in tRNA's specific for other amino acids. It would be easy to imagine that this common feature is the anti-codon specific for valine. However, there is no evidence that this is the case. Each tRNA molecule possesses about 80 nucleotides so there is abundant possibility for the existence of many other highly specific combinations of nucleotides. It should be noted that five yeast tRNA's have had their structures completely elucidated (alanine, Holley *et al.*, 1965; serine I and II, Zachau *et al.*, 1966; tyrosine, Madison *et al.*, 1966; phenylalanine, RajBhandary *et al.*, 1966). In every case, the suggested anti-codon contains one of the so-called minor bases and is very remote from the adenosine residue which is to be aminoacylated. Moreover, among the four sequences shown in Figure 4, one can detect only a few patterns that are present in every case, *i.e.*, C-C-A at the receptor end and A-G-C-Ψ-T-G in the left hand loop. In all other areas, there are a sufficient number of differences to permit the possibility of specific enzyme binding. If the clover leaf structures shown are correct, one would expect that in the paired stretches, the bases would be turned inward and inaccessible to enzyme recognition leaving the non-pairing stretches accessible. There are abundant differences even there. To date no sequences have been established for non-yeast tRNA's, but it is obvious that some differences must exist, for *E. coli* tRNA contains substantial amounts of thiouridine which is not found in yeast tRNA.

One must conclude that all tRNA's specific for a particular amino acid contain one or more features in common and that variations in the ability to be aminoacylated with heterologous enzymes reflect either differences in the binding of tRNA to enzyme or binding in a non-productive mode. In either case, these variations must be due to structural variations outside the enzyme recognition area of the tRNA and to differences in the corresponding parts of the enzymes.

Surprisingly, the cross reactions are not at all reciprocal. Thus, starfish leucine enzyme esterifies *E. coli* tRNA excellently while *E. coli* leucine enzyme appears inert towards starfish tRNA. Both yeast enzymes are active towards *E. coli* tRNA while the reverse pair do not react well. Toadfish tRNA is very active with yeast leucine enzyme while the reverse pair is sluggish.

Some generalization may be valid. Both of the *E. coli* ligases discriminate strongly in favor of homologous (*E. coli*) tRNA while *E. coli* tRNA frequently reacts well with ligases from other sources. Species specifically in these reactions appears to reside more in the enzymes than in the tRNA's, perhaps a consequence of great inflexibility of some of the enzymes.

SUMMARY

1. Amino acid activating enzymes (aminoacyl tRNA ligases) specific for valine and leucine tRNA have been partially purified from four widely differing phyla, namely yeast, *E. coli*, starfish and toadfish.

2. The rates of aminoacylation of the four tRNA's have been determined using both the homologous enzyme and the three heterologous enzymes. In most cases there was appreciable cross-reaction, the *E. coli* enzyme and tRNA being in general least disposed to cross-react with the others.

3. In some cases the heterologous pair react more rapidly than the homologous. In one case this has been established as being a consequence of a much higher V_{max} that overcomes a poorer enzyme tRNA association.

4. Both homologously and heterologously esterified tRNA's appear to be homogeneous as measured by the kinetics of hydrolysis.

5. We interpret these observations as indicating that all tRNA's specific for a particular amino acid have at least one common polynucleotide sequence, probably in an area of the chain which is not base-paired. The marked differences in the rates of esterification and in the rates of hydrolysis of the aminoacyl tRNA's reflect other structural variations in tRNA's that do not include the enzyme recognition site. In particular *E. coli* enzymes are least able to adapt themselves to heterologous tRNA's.

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ISOLATION AND COMPOSITION OF A LOW DENSITY LIPOPROTEIN FROM THE EGGS OF ARBACIA PUNCTULATA[1]

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High and low density lipoproteins have been isolated from the egg cells of vertebrate species (Wallace, 1965; Fujii, 1960). Relatively few studies of invertebrate egg lipoproteins have been made. Wallace, Walker and Hauschka (1967) have isolated high density lipoproteins from the eggs of six species of crustaceans but no low density lipoproteins have been reported from invertebrate eggs. The present work describes the isolation and analysis of a water-soluble low density lipoprotein[2] from sea urchin eggs which contains 74% of lipid and has a molecular weight of about 10×10^6. This protein bears a remarkable resemblance to the low density lipoproteins of mammalian plasma.

MATERIALS AND METHODS

The purple sea urchin, *Arbacia punctulata*, was collected during the months of July and August in the waters of Woods Hole. Eggs were obtained by injection of 0.5 M KCl into the body cavity adjacent to the lantern and were centrifuged twice in filtered sea water to remove the jelly coat.

The lipoprotein was isolated by mixing the eggs gently for 30 minutes at 0° with 20 volumes of 0.05 M sodium phosphate, pH 6.8. All subsequent operations were carried out at 0–10°. The mixture was centrifuged for 40 minutes at 100,000 g and any material floating to the top of the tube was removed. The remaining clear supernatant fluid was adjusted to a solvent density of 1.21 by the addition of NaBr and centrifuged overnight at 100,000 g. The top 10% of each tube was then harvested, a simple matter because of the color imparted to the lipoprotein by echinochrome. The combined top fractions were then diluted with 4 volumes of NaBr, density 1.21, and re-centrifuged overnight. The resulting top fractions constituted the final purified product. Before analysis, the lipoprotein was dialyzed against at least 2 changes of solvent, generally 0.1 M NaCl. Generally, prolonged storage was avoided but 1:10,000 merthiolate was added if preparations were to be kept longer than a week in the refrigerator.

After extraction with chloroform-methanol by the method of Folch *et al.*

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[1] This work was supported by U.S.P.H.S. grant HE-05285. Analytical ultracentrifugation was carried out by Mr. L. Robinson Hyde whose assistance is gratefully acknowledged. A preliminary report of this work has been made (Marsh, 1965).

[2] High density lipoproteins are here arbitrarily defined as those of hydrated density greater than 1.1 and less than 1.2, while low density lipoproteins are defined as those with densities less than 1.1 and greater than 1.01.

(1957), total lipid determinations were made by the method of Marsh and Weinstein (1966), using tristearin as a standard. Protein was measured by the Lowry method (1951) with bovine plasma albumin as a standard. Phospholipids were determined by Bartlett's method (1959) and chromatography of the phospholipid fraction was carried out as described by Abramson and Blecher (1964). Total protein-bound carbohydrate was measured on the lipid-free protein after washing the precipitate with cold 5% trichloroacetic acid to remove chloride ions. The protein precipitate was then heated for 2 hours at 100° in 1 N H_2SO_4 and the carbohydrate content of the extract measured by the anthrone method (Ashwell, 1957) using galactose as a standard. Additional extraction of the precipitate or treatment with 2 N HCl for 3 hours at 100° did not significantly increase the carbohydrate yield. The neutral carbohydrate fraction contained 96% mannose and 4% arabinose as determined by gas-liquid chromatography of the alcohol hexacetates (Sawardeker, Sloneker and Jeanes, 1965). The amino acid composition of the delipidized protein was measured with an amino acid analyzer after hydrolysis in 6 N HCl for 23 hours at 110° in a sealed tube. Tryptophan was determined colorimetrically (Opienska-Blauth, Charezinski and Berbec, 1963) on a solution of the delipidized protein in 0.1 N NaOH. The fatty acid composition of the lipid moiety was determined by gas-liquid chromatography of the methyl esters after saponification in methanolic KOH (James, 1960). Identification of the acids was made by comparison of the retention times with known standards. Free and esterified cholesterol was measured by the method of Sperry and Webb (1950).

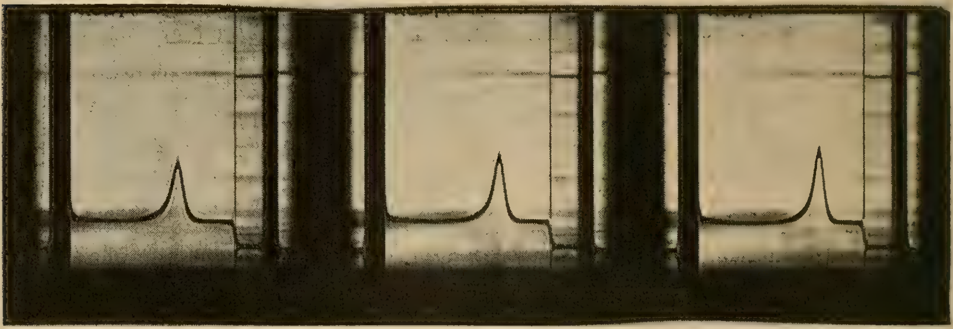

FIGURE 1. Sedimentation of *Arbacia* egg low density lipoprotein in 0.03% NaBr, pH 6, at 42,040 rpm. Sedimentation is from right to left, the interval between pictures is 4 minutes, and the bar angle 60°.

For the determination of molecular weight, the flotation rate (Sf) was measured in 24% NaBr (density 1.154 at 24°). The diffusion constant was measured in the same solvent at 6° in the electrophoresis cell of the Beckman-Spinco apparatus. No extrapolation to zero concentration was made in the measurement of Sf and D. However, the concentration employed was 0.5% and according to the data of Wallace, Walker and Hauschka (1967), the correction would be of the order of 2%. An approximate value for the partial specific volume (0.915) was obtained by determining the density of a solution of NaBr in which about

TABLE I

Lipid-to-protein ratio of Arbacia egg lipoprotein

Preparation number	Lipid/protein ratio
1	3.20
2	2.84
3	2.29
4	2.71
5	3.10*
6	2.65
Mean ± S.E.M.	2.80 ± 0.14

* Measured 24 hours after fertilization. In preparation 4, the method of determining total lipid was checked by gravimetric analysis and the resulting lipid/protein ratio was 2.78.

half the molecules sedimented and half floated at the same time at 59,780 rpm in the analytical ultracentrifuge. It was estimated that the figure for partial specific volume was only accurate within ±20%. With this information and appropriate temperature corrections, the molecular weight was calculated by the Svedberg equation.

RESULTS

The isolated lipoprotein appeared free of ordinary proteins. When floated in the analytical ultracentrifuge at density 1.2, no sedimenting protein was seen. When dialyzed against water, the protein sedimented as a single peak with an $S20_1W$ of 15.2 (Fig. 1). The lipid-to-protein ratios of 6 different preparations are given in Table I. When subjected to column chromatography on Sephadex G-200, the lipoprotein emerged with the solvent front, indicating a molecular weight higher than about 500,000, and no residual protein was eluted from the column. On disc electrophoresis with 5% polyacrylamide gel, the lipoprotein failed to enter the gel, another indication of high molecular weight. No protein was found in the gel proper after staining. Electrophoresis on cellulose acetate in 0.05 *M* barbital buffer at pH 8.6 revealed a single fairly broad band which migrated 1 cm. per hour towards the anode, a migration rate one-half that of a control sample of bovine plasma albumin. The lipoprotein was not retained on a column of carboxy-methyl Sephadex at pH 6.0, indicating an isoelectric point below that pH.

TABLE II

*Lipid composition of Arbacia egg lipoprotein**

Lipid class	% of Total lipid
Neutral lipid	62.8
Phospholipid	28.0
Sterol	8.2
Sterol ester	1.0

* The values shown are averages of duplicate determinations on two separate preparations.

Table III

*Composition of Arbacia egg lipoprotein**

Component	% of total weight
Protein	28.5
Lipid	65.2
Carbohydrate	5.8
Echinochrome	0.5
Protein-bound P	Absent

* Preparation 4 of Table I. Echinochrome was measured spectrophotometrically. Protein-bound P was measured after sulfuric acid-hydrogen peroxide digestion.

Under the conditions of isolation, the lipoprotein accounted for 70–90% of the lipid in the supernatant after the initial centrifugation in 0.05 *M* buffer. In two experiments, the yield of lipoprotein was 15.7 and 17.2 mg. of lipid per ml. of packed eggs, which accounts for approximately ⅓ of the total lipid of the cell. The overall lipid composition is given in Table II.

A complete analysis of one lipoprotein preparation is given in Table III. No protein-bound phosphorus was detected (Table III).

The fatty acid composition of the total lipid is shown in Table IV and the amino acid composition of the protein is shown in Table V. For purposes of comparison, the amino acid composition of human low density (B) lipoprotein as calculated from the data of Margolis and Langdon (1966) is also given.

No quantitative analysis of the lipid classes present was made, but judging by the extent of phosphate color given on thin layer plates, the major phospholipid was phosphatidyl choline, with phosphatidyl serine and ethanolamine present in lesser amounts. The lysophosphatides of these were also detected, along with

Table IV

Fatty acid composition of Arbacia egg lipoprotein lipid

Fatty acid	Relative retention time*	% of Total
12:0	0.18	0.2
14:0	0.31	7.1
14:1	0.41	3.1
16:0	0.55	25.4
16:1	0.68	14.7
?	0.83	0.5
18:0	1.00	6.8
18:1	1.19	9.7
18:2	1.57	4.2
20:0	1.82	2.6
18:3	2.08	10.9
?	2.42	2.2
22:1	3.90	11.2
?	5.40	1.3

* Relative to methyl stearate, on a 4 ft. column of polyethylene glycol succinate at 167° and 7.5 lbs. pressure of argon. The Pye instrument with an argon ionization detector was used.

TABLE V

*Amino acid composition of Arbacia egg lipoprotein, compared with human plasma low density lipoprotein**

Amino acid	% of Total *Arbacia* lipoprotein	Human B-lipoprotein*
Aspartic	11.1	10.9
Threonine	6.8	5.8
Serine	7.1	6.6
Glutamic	10.8	14.0
Proline	2.6	3.0
Glycine	2.6	2.6
Alanine	5.4	5.4
½ Cystine	6.1	0.6
Methionine	—	2.1
Isoleucine	5.8	5.8
Leucine	9.5	9.5
Tyrosine	3.1	4.1
Phenylalanine	6.8	6.6
Lysine	5.3	8.0
Histidine	4.8	3.0
Arginine	8.6	4.8
Tryptophan	1.5	0.9

* Data for human plasma B-lipoprotein calculated from the data of Margolis and Langdon (1966).

phosphatidic acid, but these were all believed to be breakdown products since no special precautions were taken. There were two unknown spots present. Although it had the same R_f as tristearin in two solvent systems on thin layer chromatography, the neutral lipid was not positively identified as triglyceride, since marine lipids are known to contain alkoxydiglycerides.

Some physical constants of the *Arbacia* egg lipoprotein are given in Table VI, and it can be seen that the molecule has a very high molecular weight, of the order of ten million.

DISCUSSION

Hen's egg yolk contains three main lipoproteins, a low density lipoprotein; lipovitellin (a high density lipoprotein as we have defined it) and a lipovitellin-phosvitin complex (Fujii, 1960). The lipoproteins of several classes of marine eggs described by Fujii (1960) and the crustacean egg lipoproteins isolated by

TABLE VI

*Some physical constants of Arbacia egg lipoproteins**

$S_{20,W} = 15.2$
$D = 0.79 \times 10^{-7}$ cm.^{2}/sec. in 24% NaBr at 6°
$S_f = 20.1$ in 24% NaBr at 24°
Mol. wt. $= 10 \times 10^6$

* See texts for details of measurements.

Wallace *et al.* (1967) and by Zagalsky *et al.* (1967) are high density lipoproteins. With the isolation of a low density lipoprotein in the present work, it now appears that both vertebrate and invertebrate eggs, as well as mammalian plasma, contain lipoproteins of both high and low density.

The *Arbacia* egg low density lipoprotein described here has striking similarities to that of human plasma. Its molecular weight falls in the same range of from 2 to 20 million. It is not a phosphoprotein, as are the hen's egg lipovitellins. In this respect, it resembles the crustacean egg lipoproteins. Like human plasma lipoproteins, it contains covalently bonded carbohydrate. No report of the carbohydrate content of the crustacean egg lipoproteins was made by Wallace *et al.* (1967) nor were the other egg lipoproteins isolated by Fujii (1960) analyzed for carbohydrate. Zagalsky, Cheesman and Ceccaldi (1967) have shown that the carotenoid-containing lipoprotein of the eggs of *Plesionika edwardsi*, molecular weight of about 600,000, contains 27% lipid and that the lipid-free protein contains 2.6% of carbohydrate. Mannose and glucosamine were shown to be present. The amino acid composition of the *Arbacia* egg low density lipoprotein is strikingly similar to that of human B-lipoprotein, except for the absence of methionine. Both proteins do not have large amounts of non-polar residues, and the implications of this have been discussed by Margolis and Langdon (1966).

The lipid composition again resembles that of human B-lipoprotein, since the neutral lipids made up the main component (Table II). The fatty acid composition is not remarkable, resembling marine lipids in its content of C-20 and C-22, especially the latter.

Recently, Malkin, Mangan and Gross (1965) have described a crystalline 27S protein isolated from *Arbacia* eggs, having a molecular weight of 894,000 which they believe to be derived from the breakdown of yolk granules. No information concerning lipid content was given. It is possible that their protein is a high density lipoprotein, similar to that having a molecular weight of 2.5×10^6 isolated by Fujii (1960) from cuttle-fish eggs.

Removal of lipid from the *Arbacia* lipoprotein by organic solvents at low temperature (−20°) renders the protein insoluble in water. This is quite typical behavior for human plasma B-lipoprotein. Although on one occasion it appeared that the resulting protein was soluble in 6 *M* urea (Marsh, 1965), we have not been able to repeat this observation, probably owing to denaturation problems. The minimal number of peptide chains has not been determined by end group analysis as yet.

The similarity between the storage low density lipoprotein of *Arbacia* eggs and human plasma low density lipoproteins leads to the suggestion that the latter may have evolved from storage lipoproteins. Future studies of the comparative biochemistry of egg cell lipoproteins should lead to much valuable information about water-soluble lipoproteins in general.

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THE DEVELOPMENTAL POTENTIALITY OF THE LIVER-RNA-TREATED POSTERIOR PRIMITIVE STREAK IN THE CHICK EMBRYO[1]

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Previous experiments have shown that RNA-treated posterior third of the primitive streak, here abbreviated to TPS, was capable of developing into highly organized kinds of tissue. The type of tissue produced varied according to the kind of RNA used (Sanyal and Niu, 1966). Brain RNA induced the formation of neural tissue. Kidney and heart RNAs seldom induced neural formation but caused the TPS to self-differentiate into tubular and vesicular structures, respectively. On the other hand, liver (L) RNA not only induced epiblasts to become neural tissue but also made the TPS self-develop into some organized but as yet unidentifiable tissue. The presence of the latter tissue indicated the need for prolonged cultivation. In this respect, the technique (New, 1955) used was limited. In the experiments now to be reported the L-RNA-treated TPS was implanted into the coelom of 2–2 ½-day chick embryo (Hara, 1961). The choice of intracoelomic grafting was made on three grounds: (1) the implant would have the time needed to develop its acquired capability, (2) the implant would be in contact with host's incompetent cells and thus would only undergo self-differentiation, and (3) the implant would be made to the host at an early stage when visceral organs, particularly endodermal derivatives, had not yet appeared. Both L-RNA and L-protein (serum albumin, abbreviated S.A.) were employed to treat the TPS. The aim of this paper is to present data showing that: (a) S.A. has no effect on the development of TPS, (b) L-RNA stimulates both growth and differentiation, and (c) RNase-treated L-RNA reduced gut formation but promotes growth of the implants and development of feather buds even better than RNA.

MATERIALS AND METHODS

Preparation of TPS

Fertilized eggs of White Leghorn chickens were obtained from Shaw Hatchery, West Chester, Pa. They were incubated for 15–18 hours at 38° C. in a forced draft incubator. The primitive streak of the blastoderm, 1.2–1.6 mm. in length and 0.2 mm. in width, was excised and stretched on agar dishes containing modified

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[2] State Department Scholar from Finland for the year 1965–1966.

Locke solution (a mixture of 100 ml. NaCl (9.43 g./l.), 3.7 ml. KCL (1.2 g./100 ml.), 2.1 ml. $CaCl_2$ (2.36 g./100 ml) and 1 ml. glucose (0.2 g./100 ml.). The posterior third, 0.4–0.5 mm., was cut off and immediately transferred to the Locke solution. Accumulated pieces were divided into groups and transferred to the Locke solution (2 ml.) with or without S.A. (4 mg./ml.), L-RNA ($O.D._{260 \ m\mu}$ 80/ml.) or RNase-treated L-RNA (1 mg. RNase in 2 ml. with O.D. 160). They were kept in a cold room (2–4° C.) with an occasional stir for 15–18 hours (overnight). Bovine S.A. (fraction V) was obtained from Armour and pancreatic RNase through Worthington.

Implantation of TPS

Both untreated (control) and treated TPS were implanted into the coelomic cavity of 2–2 ½-day-old chick embryos according to the procedure of Hara with a minor modification, namely, when the host was slightly older, the graft was implanted into the posterior rather than anterior end of the cavity. This modification was made for the sole purpose of increasing the number of the recipient embryos. There was no difference in the rate of recovery, growth and differentiation between the anterior and posterior grafts. All implants were harvested at the 7th–9th day and fixed in Bouin's solution. Sections were 8 μ thick and stained with hematoxylin and eosin.

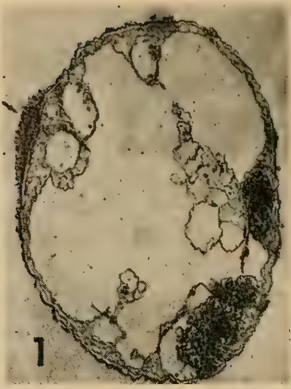
Preparation of RNA

RNA was isolated from calf liver. Thanks to Messrs. Collis and Owen of Cross Brothers Meat Packing Co., the liver was quickly removed from the slaughtered calf, sliced and put in ice-cold sucrose solution (0.25 M + $CaCl_2$, 0.003 M). The procedure of isolation has been published elsewhere (Hillman and Niu, 1963).

The glycogen of the RNA preparation was removed by Spinco centrifugation (30,000 RPM for 30 minutes) and the phenol extracted by repeated ether (or ethanol) washings, or by bubbling N_2 through. The UV absorption spectrum of L-RNA was typical of nucleic acids with maximal absorption at 260 mμ and minimal 230. The ratio of A_{260}/A_{230} exceeded 2 and that of 280/260 varied between 0.45 and 0.5. Contamination of protein, DNA and polysaccharides was routinely estimated. The amount was less than 1%. The test with orcinol yielded a green color. When a preparation did not meet the criteria given here, it would not be used for experimentation.

For functional studies of RNA, it is of prime importance to isolate the RNA within the shortest time and to use it as soon as possible. Lyophilization and storage for longer periods in a deep freezer resulted in the loss of activity except the property pertaining to growth.

Digestion of L-RNA was carried out in 2 ml. of Locke solution containing RNA and 1 mg. of RNase. The mixture was incubated at 37° C. for 1 hour. This resulted in an increase of O.D. by 35%. To an aliquot of the sample, an equal volume of ice-cold 4% perchloric acid was added. The acid-precipitable material was the undigested portion of RNA. It amounted to 15–20% of the original.



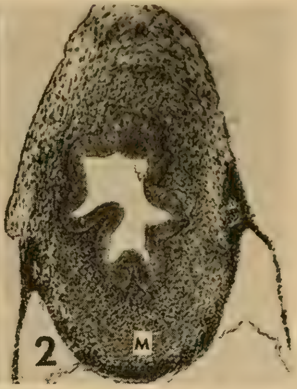


FIGURE 1. A section through the implant of control. Note the presence of endodermal epithelial lining on the outer surface (at 10:00 o'clock) and two islands of blood cells on the lower left. × 120.

FIGURE 2. A section through the L-RNA-treated implant showing intestinal vesicle. M, circular muscle. × 120.

RESULTS

Development of the Untreated TPS (controls)

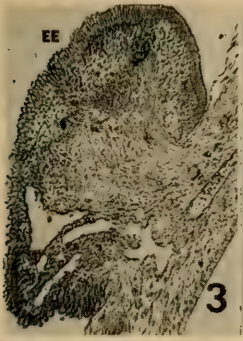
A total of 60 fragments was excised. After being kept in the modified Locke solution at 2–4° C. for 15–18 hours, they were implanted into the coelomic cavity. The explants were loose and fell readily into tiny pieces, thus causing some difficulty during the process of implantation. Histological examination revealed, however, that cells of the central mass were normal and those in the outermost layer became swollen or even broken. Of the 60 controls, 23 (38%) were recovered.

The implants are either free or attached to the host tissue. Histological examination of the controls showed that the structures produced were limited to tissue with or without epidermal and/or endodermal epithelial lining. There was no appreciable difference between the untreated (control) and the S.A.-treated series, thus showing that the slight damage to the outermost layer of cells, caused by keeping the explants overnight in the cold room, had no adverse effect on their developmental potentiality. To further demonstrate this point,

TABLE I

Developmental potentiality of the control posterior primitive streaks

Conditions of explant	Growth		Differentiation				
	Attachment to host	Size increased 3 or more times	Total	Epidermal	Epidermal-feather buds	Endodermal	Gut
Fresh controls (57)	14	3	7	5	1	5	0
Aged controls (60)	14	6	9	7	3	7	1



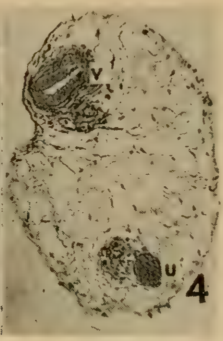


FIGURE 3. A section through a tiny portion of a big skin vesicle, developed from a L-RNA-treated TPS. The attached mass of connective tissue (see also Fig. 7) is covered by a distinctive layer of endodermal epithelium, EE. × 100.

FIGURE 4. A section through a poorly developed vesicle of the L-RNA-treated implants. V, intestinal vesicle and U, unidentified tissue. × 100.

10 anterior primitive streaks including Hensen's node were kept in the cold room for 15–18 hours. The explants showed similar loose consistency as noted in the TPS. All developed into large or very large grafts containing skin, brain, intestine, cartilage, etc.

Usually the implants were small but compact. When somewhat larger, they contained some connective tissue, blood (Fig. 1) and occasionally smooth muscle fibers. In cases when they were attached to the host, they were covered with poorly organized, vacuolated, spherical, or more or less elongated cells. These cells formed a definite layer in some cases and had the appearance of intestinal epithelium (arrow in Fig. 1).

TABLE II

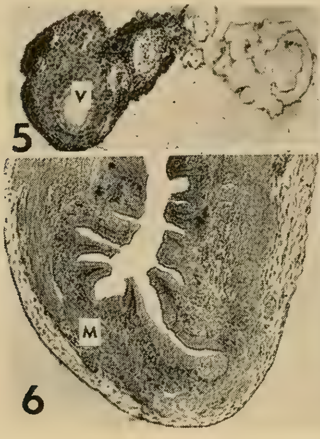
Growth and differentiation of the posterior third primitive streak treated with and without liver protein (S.A.) RNA and its hydrolytic products (A = total number, B = percentage of the total & C = percentage of the recovered)

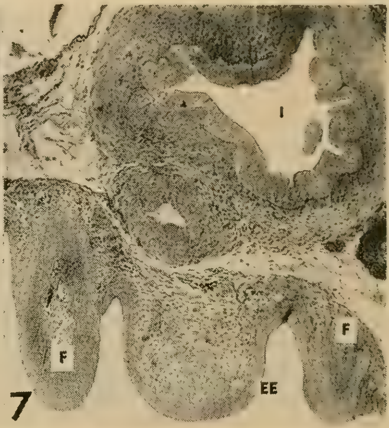
Experimental series	Untreated (control)			Treated with S.A.			Treated with RNA			Treated with RNase-digested RNA		
	A	B	C	A	B	C	A	B	C	A	B	C
1. Implants	60	—	—	33	—	—	46	—	—	29	—	—
2. Recovered	23	38	—	13	39	—	27	59	—	23	79	—
3. Attached to host tissue	14	23	61	8	24	62	21	46	76	12	41	52
4. Size increased 3 or more times	6	10	26	2	6	15	16	35	59	14	48	61
5. Differentiation	9	15	39	6	18	46	20	43	74	14	48	56
6. Epidermal	7	12	30	3	9	23	14	30	52	9	31	39
7. Epidermal with feather buds	3	5	13	2	6	15	9	20	33	9	31	39
8. Endodermal	7	12	30	5	15	29	17	37	63	7	24	30
9. Gut	1	2	4	2	6	15	9	20	33	2	7	9

TPS were also implanted immediately into the coelomic cavity after extirpation. Of the 57 fresh controls, 37 (65%) were recovered which is superior to the 38% of the aged controls mentioned above. However, when the recovered implants were examined for growth and differentiation, the aged controls were slightly better than the fresh controls (see Table I). Although the reason for this improvement is not known, the observation undoubtedly adds support to the contention that slight damage to the explants had no adverse effect on their developmental potentiality.

Development of the S.A.-treated TPS

Thirty-three explants were kept in Locke solution with S.A. added. At the time of implantation, they were compact and looked healthy. However, the growth and differentiation of this series were remarkably similar to those of the control (Table II). It seems, therefore, that the developmental potentiality of the control and S.A.-treated TPS is similar and thus considered to be intrinsic.





FIGURES 5 and 6. Sections of two vesicles from the L-RNA-treated implants showing variations of intestinal structures. V, poorly developed intestine; M, circular muscle. × 100.

FIGURE 7. A section through a big skin vesicle obtained from RNase-treated RNA series. The lumen of the vesicle is toward the bottom. The attached mass has developed into distinctive intestinal tubes (I). EE, epidermal epithelium. I, intestinal tube, and F, feather follicle. × 120.

Development of the L-RNA-treated TPS

At the time of implantation, the explants were similar to those of the S.A. series. A total of 46 implants were made and 27 (59%) were recovered. Most of them were attached to the host. More than half showed good growth. The overall differentiation was 43% of the total. About half (14) of the available grafts (27 cases) contained epidermal epithelium. Nine of the 14 appeared in the form of large vesicles with feather buds, *i.e.* 64% of epidermal vesicles developed feather buds. Of the 27 recovered implants, 17 developed into tissue with endodermal epithelial lining (Figs. 3–5) and 9 into intestinal vesicles (Fig. 2) or well formed intestinal tubes (Fig. 6). It should be noted that differentiation of both epidermal and endodermal epithelial cells was usually found in the same graft. These grafts were well differentiated and rather large.

Development of the TPS treated by RNase-digested L-RNA

Incubation of L-RNA with RNase resulted in a loss of 85% of RNA. The RNase-treated L-RNA (RNase, RNA and its hydrolytic products) was used to treat 29 explants. Before implantation, they were compact and healthy. Twenty-three implants were recovered, thus providing the highest rate of recovery (79%) in our experimental series. The rate of growth was also the best (Fig. 7). The overall rate of differentiation and the formation of epidermal epithelium were similar to the RNA series. However, the development of endodermal epithelium and gut particularly decreased from 37% to 24% (a loss of 35%) and from 20% to 7% (a loss of 65%), respectively.

The effect of plain RNase on TPS was also tested. Seven explants were kept in Locke solution containing RNase. None of the implants was recovered after 10 days in the peritoneal cavity of the developing chick embryos.

DISCUSSION

The TPS used for implantation was taken from the region where epiblasts and hypoblasts merge into a mass of cells. They are destined to give rise to connective tissue, and epidermal and endodermal epithelium (Willier and Rawles, 1931a), but are capable of being induced to develop into tissues other than epithelial cells. However, when they were grafted into the developing blastoderm, earlier experiments showed that 72% of the implants could not be located at the site of implantation and the remaining 28% appeared as mesenchyme, seldom developing into a neural tube. In the foregoing experiments, the explants were grafted into the coelomic cavity. The rate of recovery was improved (38%, Table II). Among the chemically treated series, S.A. did not alter the rate of recovery (39%), nor development. L-RNA and its hydrolytic products increased the recovery rate up to 59% and 79%, respectively. The improvement was accompanied by better growth and differentiation.

The implant of control groups developed into connective tissue with or without epidermal epithelium and/or endodermal epithelial lining. The formation of ectodermal (feather buds) or endodermal derivative (gut) was rare. Therefore, the developmental potentiality of TPS was better realized in the coelomic cavity of 2 ½-day chick embryo than with the *in vitro* technique (New, 1955). This finding is in accord with the recent observation that the type of medium used is extremely important in the study of differentiation (Spratt and Hass, 1967).

The developmental potentiality of the control and chemically treated TPS was compared categorically in terms of total implants. It can be seen in Table II that the data of the control resemble the S.A.-treated series. Both differ significantly from RNA and RNA-digest series. The latter two series stimulate both growth and differentiation. Of particular interest is the finding that gut formation is increased by RNA, and feather bud formation can better be facilitated by the hydrolytic products than RNA itself. Feather bud is one of the ectodermal derivatives. Its frequency of development in the epidermal vesicles is 43% of the control, 66% S.A., 64% RNA and 100% RNA digest series. Furthermore the RNA digests cut down the developing rate of endodermal epithelium (from 37% to 24%) and gut (from 20% to 7%), but increase the rate of recovery (from 59% to 79%) and the number of sizable implants (from 35% to 48%). Therefore, the reduction in development of endodermal structure is related to the loss of RNA (about 85%) and the digests are responsible for the gain in feather bud formation and growth of the implants as well. The action of the latter is not specific and belongs to the same category as reported by Sengel (1964). They perhaps contribute to the enrichment of the nutrients. It may be relevant to mention here that much of the exogeneous RNA remains intact in the recipient cells (Niu, Niu and Guha, 1968) and some are being hydrolyzed which, in turn, may be responsible for the non-specific action of RNA.

Gut was found once in 60 implants of the controls and twice in 33 S.A. and 29 hydrolyzed RNA series, respectively. This low frequency could hardly be used to argue for the action of S.A. or RNA digests on gut differentiation. Instead we consider this gut-forming ability as one of the intrinsic properties of TPS. L-RNA was capable of increasing the rate of gut formation three-fold (from 6% to 20%). This amounts to 45% of the cases with differentiation.

A double check on the developmental potentiality was made by using recovered implants (see Column C of Table II). The rate of attachment (row 3) is compared with that of the growth (row 4) and differentiation (row 5) of the same series. It can be seen that the rate of growth and differentiation is notably lowered in the control and S.A. series. On the other hand, the RNA and RNA-digest series have maintained at about the same level as the attachment. The types of differentiation are derivatives of the ectoderm and endoderm. The ectodermal derivatives of the control are similar to the S.A. series (rows 6 and 7), but strikingly different from the RNA and RNA-digest series. As to the endodermal derivatives (rows 8 and 9) the rate of development is approximately the same in the control, S.A. and RNA-digest series *but* less than half of the RNA series. In other words, the RNA used in the present experiment stimulates the formation of endodermal derivative, and this function requires the intact macromolecule of RNA. The promotion of ectodermal formation by RNA is non-specific because hydrolytic products of RNA can do almost equally well.

Liver is an organ derived from the ventral diverticulum of the gut. In the chick, its development is rather complicated and requires an inducing action from the heart rudiment (Willier and Rawles, 1931b). The fact that L-RNA can indeed stimulate gut formation is a step forward in our study on the possibility of the directed formation of specific tissue (Hillman and Niu, 1963; Niu and Leikola, 1966; Sanyal and Niu, 1966). Should L-RNA carry the message of the liver (Niu, Cordova and Niu, 1961; Zimmerman, Zoller and Turba, 1963), one immediate question is the condition under which the development of gut and liver can separately be achieved. Experiments dealing with this and other related problems are in progress.

SUMMARY

The posterior third of the primitive streak was implanted into the 2 ½-day chick embryo. Both growth and differentiation of the untreated implants were similar to those of the serum albumin series. Treatment with liver RNA, however, resulted in a significant increase in the rate of growth and differentiation. When liver RNA was incubated with RNase, the products furthered the growth and feather bud formation, but reduced the differentiation of the endodermal derivatives, especially gut.

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THE BIOLOGY OF FERTILIZATION AND BROOD PROTECTION IN SPIRORBIS (LAEOSPIRA) MORCHI[1]

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The described species of the serpulid genus *Spirorbis* are hermaphroditic and exhibit brood protection. Alexander Agassiz (1866) appears to be the first investigator to offer information on the biology of fertilization in the genus. Agassiz, who examined *Spirorbis spirillum,* claimed that the embryos reached quite an advanced stage within the body of the parent before the brood sac was deposited within the cavity of the tube. Fewkes (1885) examined what he thought to be *Spirorbis borealis* and observed that the eggs were laid in strings within the parental tube; however, he was not able to confirm Agassiz' claim that development initially occurs within the body of the parent. According to Schively (1897), who worked with what she believed to be *Spirorbis borealis,* the eggs pass into the body cavity and then into the operculum where fertilization takes place and a capsule is secreted. Schively further claimed that once the egg capsule was secreted, the capsule passed through an opening in the operculum and was placed in the "mid-dorsal furrow." It seems quite likely that both Fewkes and Schively were working with *Spirorbis spirillum* and not *Spirorbis borealis.* Zur Loye (1908) believed that fertilization in *Spirorbis borealis* occurred externally within the parental tube. Abe (1943), working with a species which he identified as *Spirorbis argutus,* reported that fertilization probably took place internally. Recently, Gee and Williams (1965) have reported that fertilization in *Spirorbis borealis* and *Spirorbis pagenstecheri* occurs externally and have presented evidence that self-fertilization is possible in both species.

Brood protection in the genus *Spirorbis* takes place either within the parental tube or within a modified operculum. As far as is known, the mode of brood protection is species-specific. Thorson (1946) has claimed that *Spirorbis granulatus,* which is characterized as an operculum brooder (Caullery and Mesnil, 1897; Bergan, 1953b), broods in the tube and suggested that the mode of brood protection varies within the species; however, Thorson (personal communication) believes that he may have been mistaken as to the identity of the species. Of the two types of brood protection, opercular brood protection is the most specialized and has been considered a recent development in the evolution of the genus (Elsler, 1907; Borg, 1917; Gravier, 1923). Bergan (1953a) observed the presence of a pore in the operculum of *Spirorbis granulatus,* but was of the opinion that it was too small to allow the passage of eggs and did not pursue its significance. To date, there has been no attempt to explain how spawned oocytes are transferred to the opercular brood pouch.

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The present paper deals with the biology of fertilization and opercular brood protection in *Spirorbis* (*Laeospira*) *morchi* Levinsen. The observations on fertilization are an elaboration of those presented earlier in abstract (Potswald, 1964).

MATERIALS AND METHODS

Spirorbis morchi adults were collected periodically throughout the year, from 1960 to 1963, in Argyle Creek on San Juan Island, Washington. The animals were found on rock and shell and often in association with *Spirorbis* (*Paradexiospira*) *vitreus*. In Argyle Creek, *Sp. morchi* breeds the year around; consequently, developmental stages were always available.

In order to determine whether animals raised in strict isolation are capable of self-reproduction, individual larvae were isolated and cultured. This was accomplished by placing larvae in polyethylene ice-cube trays, one larva per cube, and allowing them to settle. Each cube was given a number, thereby allowing complete records to be kept for each isolate. The sea water used in these cultures was filtered to prevent larvae, that might be in the sea water system, from settling in the containers used to store the water, and was then allowed to stand for a minimum of three weeks before using. Such water was considered to be sperm-free and will be subsequently referred to as sperm-free sea water. The sea water was removed periodically, by means of a pipette attached to an aspirator, and replaced. A new pipette was used for each cube, thus eliminating the possibility of cross-contamination by introducing sea water from one cube into that of another. Initially, food was supplied in the form of *Nitzschia* sp.; however, the cultures fouled very rapidly even upon the addition of small numbers of diatoms. It was found that the animals maintained themselves on protozoa and bacteria present in the water so the addition of diatoms was discontinued.

Larvae used in the isolation experiments were artificially released from brood pouches. Opercular brood pouches, which contained actively moving larvae, were selected and removed with #5 watchmaker's forceps. Once removed, the opercular ampullae were torn open with forceps or size 0 insect pins. The larvae thus released showed the same behavioral responses and settled in about the same length of time as naturally released larvae.

The histological techniques used in the present study were reported previously (Potswald, 1967). In addition, the chloranilic acid method of Carr *et al.* (1961) was used for the demonstration of calcium.

OBSERVATIONS ON FERTILIZATION

Attempts at controlled fertilization

In the summer of 1961, while in residence at the Friday Harbor Laboratories, several experiments were carried out in an attempt to bring about controlled artificial fertilization. Adults with gravid female segments were removed from their tubes, individually isolated in syracuse dishes, and gently washed with two or three changes of pasteurized sea water to remove any extraneous sperm that may be adhering to the body. Male and female segments were then jabbed a few times with a size 0 insect pin which caused the longitudinal muscles of the abdomen to contract strongly and spasmodically, resulting in the release of gametes through ruptures in the body wall. After a minute or two, the oocytes were removed by means of a pipette and transferred through several changes of pasteurized sea water to prevent possible polyspermy. Periodic examination with the compound microscope revealed that in over half the animals tested, activation had been initiated, as manifested by the lifting of a fertilization envelope; however, in none of the cases observed was there germinal vesicle breakdown accompanied by polar body formation. The same results were obtained when sperm from another individual were added. In all cases, the sperm were active and the oocytes, as judged by morphological criteria, appeared to be "ripe." It is possible that physiological maturation of the oocytes precedes natural spawning by only a matter of hours as has been shown by Howie (1961) for *Arenicola marina*.

Isolation of larvae

A second approach involved the use of artificially released larvae which were individually isolated and raised in sperm-free sea water (see Materials and Methods). Seventy larvae were isolated in the spring and summer of 1961. The mortality rate was quite high during the first year and as of August, 1963, there were only 16 survivors. Of these, only four produced broods and as can be seen from the data summarized in Table I, two of the four isolates produced more than one brood.

TABLE I

Brooding record for Spirorbis morchi adults raised from larvae in isolation

Animal	Date isolated as larva	Date found brooding	Age at first brooding	No. of embryos	Remarks
I-12	6/18/61	9/06/62	15 mos.	3	Non-viable brood
I- 6	4/07/61	9/20/62	17 mos.	4	Non-viable brood
I-15	7/21/61	12/ 8/62	17 mos.	5	Non-viable brood
		2/22/63		3	Larval release & settlement
III- 4	7/03/61	9/06/62	14 mos.	4	Larval release & settlement
		10/22/62		5	Larval release & settlement
		12/13/62		?	Operculum & brood shed before completion of development
		3/09/63		?	Non-viable brood

Among the four animals which were able successfully to mature and spawn gametes, only a small number of eggs per brood were produced. This small number, and the fact that only ¼ of the survivors were able to spawn at all, can probably be attributed to nutritional factors. As mentioned earlier, attempts to supply food, in the form of diatoms, were not successful, and therefore discontinued. Obviously, settled animals can be maintained on bacteria and protozoa present in the sea water; however, only a few will be able to mature a small number of gametes under these conditions. At the time the cultures were discontinued (March, 1964), 12 of the original isolates still survived and ranged from 31 to 33 months in age. One of the isolates was sectioned and although it had the adult number of segments, all abdominal segments were completely agametic.

The cause of non-viable brood formation is not known. In the above cases, it is quite possible that mature sperm were not formed and, consequently, the oocytes not fertilized. It should be pointed out, however, that non-viable broods are often found in animals taken from the field. The fact that viable larvae, capable of normal settlement and metamorphosis, were produced from animals isolated as larvae is conclusive proof that in *Sp. morchi*, reproduction in isolation is possible. Had more effort and time been spent in finding a proper food source, it might be assumed that all of the isolates would have produced viable broods, probably over quite an expanse of time.

Isolation of adults

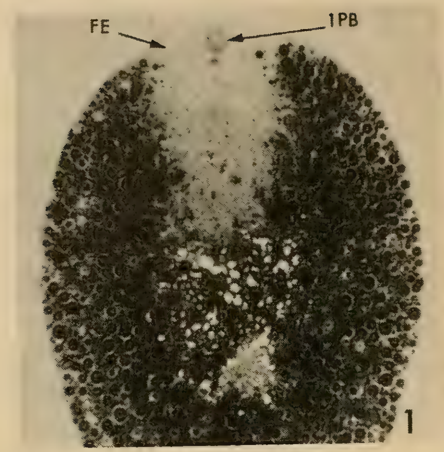
To test sperm viability, contents of male segments were artificially released, placed in filtered sea water, kept at about 12° C., and periodically examined with the compound microscope. Mature sperm remain active during the first eight

Table II

Isolation of Spirorbis morchi adults

Animal	Date of isolation	Date of spawning	Time elapsed since isolation in days	Remarks
1	6/18/62	7/05/62	17	Arrested development
2	6/18/62	6/23/62	5	Larvae released 7/24/62
*3	6/18/62	6/22/62	4	Larvae released 7/24/62
4	6/19/62	6/22/62	3	Arrested development
5	6/19/62	7/09/62	20	Arrested development
6	6/22/62	6/25/62	3	Arrested development
7	6/26/62	7/01/62	5	Larvae released 7/26/62
8	6/29/62	7/01/62	2	Larvae released 8/01/62
9	6/29/62	7/04/62	5	Arrested development
10	7/05/62	7/13/62	8	Larvae released 7/12/62
*11	7/12/62	7/26/62	14	Arrested development
12	2/07/63	2/14/63	7	Arrested development
13	2/07/63	2/17/63	10	Arrested development
*14	2/07/63	2/17/63	10	Larvae released 3/25/63
15	2/07/63	3/13/63	34	Arrested development
		4/06/63	58	Fixed 4/06/63
16	2/07/63	2/19/63	12	Larvae released 3/23/63
		3/27/63	48	Larvae released 4/25/63
17	2/07/63	3/12/63	33	Arrested development
18	2/07/63	2/13/63	6	Arrested development
		3/20/63	41	Larvae released 4/19/63
19	2/07/63	2/17/63	10	Arrested development
20	2/07/63	2/12/63	5	Larvae released 3/20/63
		3/27/63	48	Arrested development
21	2/07/63	3/20/63	13	Arrested development
22	2/07/63	3/09/63	30	Larvae released 4/13/63
23	2/07/63	3/06/63	27	Arrested development
*24	8/12/63	8/14/63	2	Larvae released 9/24/63
*25	8/12/63	9/05/63	25	Larvae released 10/17/63
		11/ 7/63	84	Non-viable
26	8/19/63	8/22/63	3	Arrested development

* Animal left in tube.



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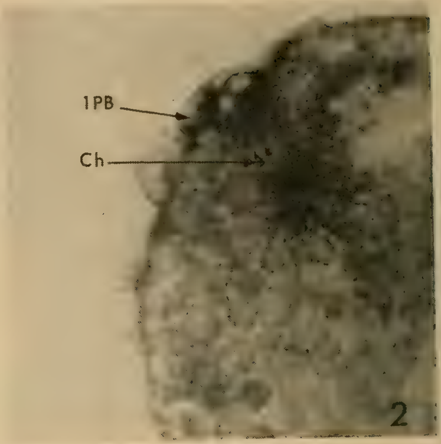

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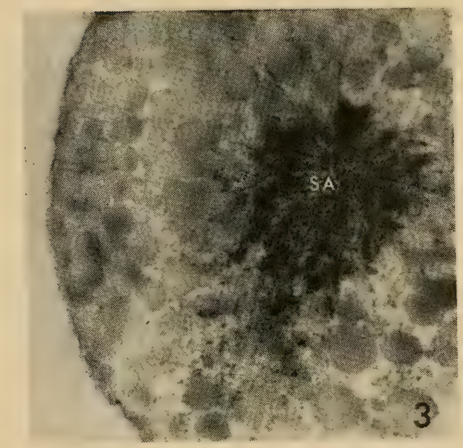

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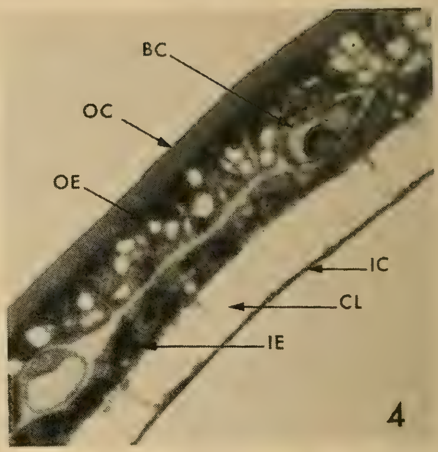
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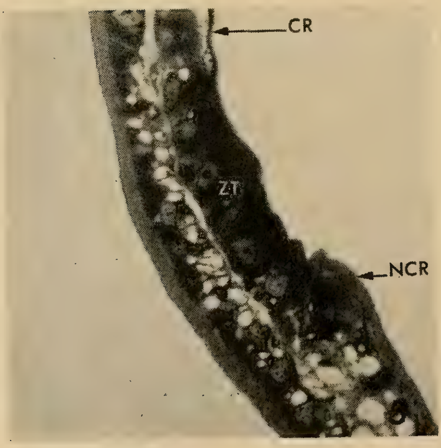

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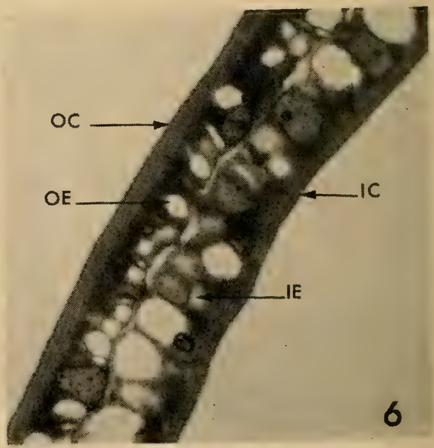

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Figures 1–6.

hours after release, but become completely non-motile within 24 hours. "Plates" of spermatids remain active for three days but start to disintegrate on the fourth day without completing metamorphosis. With these facts in mind, adult *Sp. morchi,* taken from the field and in most cases removed from their tubes, were isolated in either 10-ml. beakers or polyethylene ice-cube trays containing pasteurized sea water. Daily observations were made to determine the length of time elapsed after isolation for spawning to occur. Of 63 isolates, 26 spawned, and of these, 13 produced broods of normal larvae. The results are summarized in Table II.

It will be subsequently explained that *Sp. morchi* adults removed from their tubes are not always capable of transferring all of their spawned oocytes into the opercular ampulla, and therefore, a number, and often all, of the oocytes are spawned freely into the sea water. Development outside of the brood pouch is almost always suspended, presumably because of the growth of bacteria and protozoa which adhere to the sticky fertilization envelope. In most instances, the term "arrested" where it appears in the above table refers to such cases.

In the adult isolation experiments, newly spawned oocytes were removed intermittently from culture and examined with the compound microscope. Sperm penetration was never observed and only a partial sequence of events can be given. In the earliest stage observed, the vitelline envelope was just elevating at the animal pole as the fertilization envelope. The germinal vesicle at this time appears to be still intact and lies close to the surface under the plasma membrane of the animal pole. About 2½ hours after elevation of the fertilization envelope, extrusion of the first polar body begins and is completed in about 30 minutes. The second polar body is given off about two hours after the first and with its extrusion, the first polar body divides. In live material, fine filaments can be seen extending from the surface of the polar bodies to the fertilization envelope where they apparently attach; the filaments have not been seen in fixed material. Because of the large amount of heavily pigmented yolk, male and female pronuclei have not been observed following polar body extrusion. The fertilization envelope remains closely

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FIGURE 1. Section through a fertilized oocyte showing the lifting of the fertilization envelope (FE), and first polar body (1 PB). Epon; Richardson's stain. (300 ×)

FIGURE 2. Section through an oocyte spawned after 58 days in isolation, showing the first polar body (1 PB) and two chromosomes (Ch) arranged in second meiotic metaphase. Paraffin; haematoxylin-eosin. (750 ×)

FIGURE 3. An adjacent section to that in Figure 2 showing a sperm aster (SA). Paraffin; haematoxylin-esoin. (750 ×)

FIGURE 4. Cross-section through the calcified region of the operculum showing the outer cuticle (OC), outer epithelium (OE), inner epithelium (IE), calcified layer (CL), inner cuticle (IC), and blood capillary (BC). Epon; Richardson's stain. (1125 ×)

FIGURE 5. Cross-section through the operculum in the zone of transition (ZT) between the calcified region (CR), and the non-calcified region (NCR). Epon; Richardson's stain. (750 ×)

FIGURE 6. Cross-section through the non-calcified region of the operculum showing the outer cuticle (OC), outer epithelium (OE), inner epithelium (IE), and inner cuticle (IC). Epon; Richardson's stain. (1125 ×)

applied to the egg surface except at the animal pole where the perivitelline space is about 14 microns (Fig. 1). The above observations were made at a temperature of 12–13° C.

Five oocytes spawned after the parent had been in isolation for 58 days (animal #15, second spawning) were fixed and sectioned. Cytological examination revealed that the first polar body had been given off and the chromosomes were in second meiotic metaphase; a sperm aster was present in the cytoplasm (Figs. 2, 3). Although actual fusion of pronuclei has not been observed, it is, nevertheless, concluded that *Sp. morchi* is not only capable of reproduction in isolation but of self-fertilization. The fact that both polar bodies are given off in oocytes spawned in isolation supports this conclusion and makes it seem unlikely that parthenogenesis is occurring.

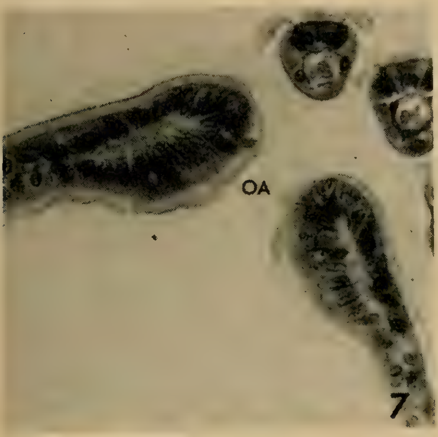
Observations on Opercular Brood Protection

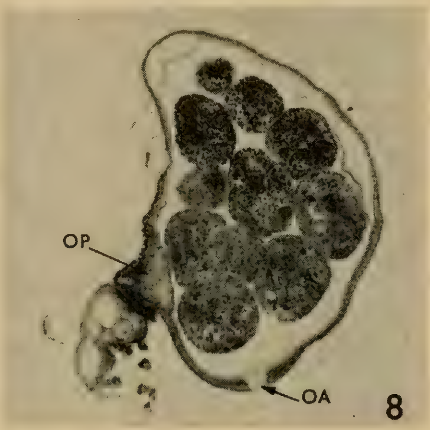
Structure of the operculum in Sp. morchi

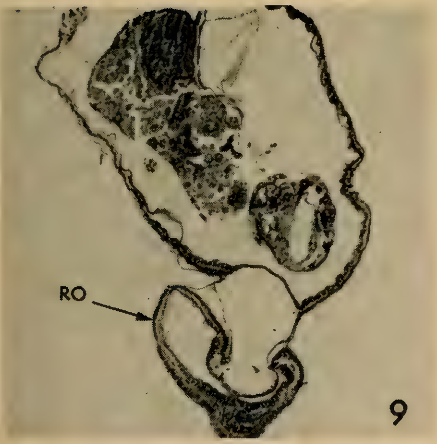
Second of the branchial tentacles in position, in relation to the dorsal midline, the operculum arises from the left branchial lobe (see zur Loye, 1908, Fig. 12). Typically, the operculum contains a spacious cavity, the ampulla, and is capped by a characteristically bilobed calcareous plate. Careful observation of a living operculum with a dissecting microscope reveals the presence of a distinct though small pore in the wall of the ampulla. It can be determined with direct lighting that, except for the ampullar side, the operculum is almost completely calcified. At the base of the operculum is a short stalk, the peduncle, which often has a groove running around its circumference. Below the groove, when present, the peduncle is swollen. This swelling is the replacement operculum and will become a functional operculum after the extant operculum is shed. The size of the operculum in mature adults can be quite variable but averages about 700 × 330 microns at its longest and widest dimensions. No attempt has been made to correlate opercular size with, for example, age classes, but it is possible that such a correlation may exist.

From the outside in, the operculum consists of an outer cuticle and epithelial layer followed by an inner epithelial layer and cuticle. There is a distinct space separating the two epithelial layers; however, a basement lamina has not been demonstrated. The histology of the calcified region of the operculum is somewhat different from that of the non-calcified portion.

In the calcified region (Fig. 4), the outer cuticle is about 2–2.2 microns thick and has a striated appearance. The outer surface of the cuticle is fuzzy, suggesting the presence of minute projections. The cuticle is strongly PAS-positive, indicating the presence of a polysaccharide, and stains blue with Heidenhain's Azan. The outer epithelial layer varies in height from 3 to 6 microns and is cuboidal. Protoplasmic processes extend from the apices of the cuboidal cells into the outer cuticle and give the latter its striated appearance. Nuclei are basal in position, and contain a single nucleolus and irregular pieces of chromatin. Several vacuoles are located within each cell of the outer layer but their significance is not known. Separating the outer epithelial layer from the inner epithelial layer is a space of about 3 microns which widens to about 6–7 microns at the sites of blood capillaries which penetrate between the two tissue layers. The inner epithelial layer is about half as thick as the outer and contains few vacuoles. Nuclei of the inner layer are more elliptical in shape than those of the outer layer. The inner cuticle is about 1 micron thick and very dense; separating it from the inner epithelium is a space which varies from 6 microns along the sides of the operculum to about 28 microns at its apex. Coarse protoplasmic processes extend from the inner epithelium across the space and attach to the cuticle. Sections of opercular brood pouches fixed in







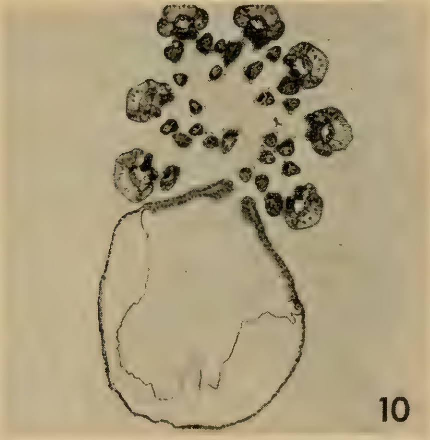


FIGURE 7. Cross-section through the opercular aperture (OA). Note that the epithelium surrounding the aperture is columnar. Paraffin; iron haematoxylin. (400 ×)

FIGURE 8. Sagittal section through an operculum containing a brood. Note the opercular aperture (OA), and opercular peduncle (OP). Paraffin; iron haematoxylin. (75 ×)

FIGURE 9. Sagittal section through an operculum containing a moribund brood. Note the developing replacement operculum (RO). Paraffin; haematoxylin-eosin. (75 ×)

FIGURE 10. Cross-section through the branchial crown showing that the opercular aperture opens to the center of the crown. Paraffin; haematoxylin-eosin. (75 ×)

neutral formalin and treated with the chloranilic acid method reveal that the space between the inner epithelium and cuticle is the site of calcification. The surface of the inner epithelial layer, facing the cuticle, gives a very strong reaction and presumably it is this layer that is responsible for calcium secretion.

There is a sharp zone of transition between the calcified and non-calcified regions of the operculum (Fig. 5). In the non-calcified portion, the outer cuticle and epithelium are similar to those of the calcified region (Fig. 6). The space separating the outer epithelium from the inner epithelium averages 1–3 microns and there are fewer blood capillaries than are found in the calcified region. The inner epithelium varies from about 7 to 9 microns in thickness and is also cuboidal in appearance. Nuclei are tall, having a height almost equivalent to that of the entire cell. One or more large intracellular vacuoles are present, but often disappear when the operculum contains a brood. In both epithelial layers of the calcified and uncalcified regions alike, the cells contain within their cytoplasm several dark-staining spherical bodies, each of which encloses a less dense internum. The dark-staining bodies are removed in the solvents used in routine paraffin embedding which suggests that they are lipid in composition. The inner cuticle of the uncalcified region is like the outer except the surface is less fuzzy and there is a thin membrane-like layer applied to it.

Around the opercular aperture the epithelial layers become considerably thickened (Fig. 7). The cuboidal nature of both outer and inner epithelium changes to columnar. The height of the outer cells averages about 13 microns and that of the inner about 22 microns. Nuclei are elongated in shape and the cytoplasm is almost completely devoid of vacuoles. The interspace between outer and inner epithelial layers is obliterated. Muscle and nerve fibers are not demonstrable.

The outer cuticle and epithelium of the operculum are continuous with those of the peduncle (Fig. 8). The inner cuticle and epithelium forms the floor of the operculum and is not continuous with any portion of the peduncle; consequently, the only opening into the opercular brood pouch is by way of the opercular pore or aperture. There is a basement lamina below the columnar epithelium of the peduncle and also below the inner epithelium of the operculum where the latter forms the roof of the peduncle. Composing the core of the peduncle are longitudinal muscle fibers and blood capillaries embedded in an ill-defined connective tissue containing fibers which stain blue with Azan. According to Hanson (1949) in the peduncle and branchial tentacles of *Sp. corrugatus* and *Sp. militaris* three nerves can be demonstrated using the Azan method; however, in the present material, using the same technique, only one nerve (the internal branchial nerve, using Hanson's terminology) could be demonstrated. At the top of the peduncle, or base of the operculum, muscle fibers can be observed to penetrate between the inner and outer epithelial layers of the operculum, but they can not be followed for any distance. As mentioned earlier, there is often a groove running around the circumference of the peduncle. This may properly be referred to as the zone of detachment because it is here that the operculum becomes disconnected from the peduncle when it is periodically shed. A new or replacement operculum makes its appearance below the old operculum and is generally well-formed before the latter is shed (Fig. 9). Experimental extirpation of the operculum results in its renewal in the same position.

Brooding behavior in Sp. morchi

The primary or first operculum formed at metamorphosis of the larva does not serve as a brood pouch but is replaced by a secondary operculum which does serve in that capacity. This conclusion is based on observations made on animals raised from isolated larvae. It cannot be stated exactly how many consecutive broods an operculum can accommodate before it is replaced, but it is certainly at least three. As pointed out by Gravier (1923), opercular replacement is not peculiar to operculum brooders but also occurs periodically in species which brood in the tube. Based on counts made on 115 broods, the average number of embryos per brood in *Sp. morchi* is 15; however, within a population, a range of from 3 to 45 embryos per brood is not unusual. The embryos are not surrounded by a common membrane within the operculum as described in *Sp. granulatus* by Bergan (1953a).

On three different occasions larval release was observed in the laboratory. Larvae emerge *via* the opercular pore which opens wide enough to allow the simultaneous release of two larvae at a time. After release of the larvae, the pore appeared to close somewhat but it could not be ascertained whether or not this closure was complete. In all three cases, within 12 hours of larval release a new brood had been deposited in the operculum and the opercular pore was tightly closed. From these observations, it is not possible to determine whether the pore opens actively during larval release or merely passively as the result of force applied by

TABLE III

Number of eggs spawned free and/or successfully transferred to the brood pouch in Spirorbis morchi adults removed from their tubes

Animal	Numbers of eggs spawned	Numbers of eggs placed in brood pouch	Numbers of eggs spawned free into the sea water
1	8	0	8
2	12	3	9
3	37	18	19
4	13	2	11
5	21	0	21
6	5	5	0
7	16	7	9
8	15	0	15
9	10	0	10
	11	0	11
10	5	0	5
	8	0	8
11	4	0	4
12	7	0	7
	8	6	2
13	13	0	13
14	13	0	13
	3	0	3
15	8	0	8
16	6	6	0
17	7	4	3
18	9	1	8

the moving larvae; however, it is certain that the pore closes after receiving a brood and this would seem to suggest an active process. It should be pointed out that in a virgin operculum the pore is tightly closed prior to deposition of the first brood and this would suggest that opening, like closing, of the pore is under active control.

Earlier it was mentioned that adults removed from their tubes are inefficient in transferring spawned eggs into the opercular brood pouch. In most instances, counts of the number of eggs spawned free and the number placed in the opercular brood pouch were made. These data are tabulated in Table III.

In all cases, the oocytes spawned free into the sea water were activated and commenced development. Failure to transfer spawned oocytes into the brood pouch has never been observed in control animals left in their tubes.

Although egg transfer has never been observed, a reasonable hypothesis can be constructed based upon the aforementioned observations. First, it has been established that the only entrance into the brood chamber is *via* the opercular pore; consequently, the oocytes must be spawned to the outside and then transferred into the operculum. The opercular pore, in some unexplained way, is capable of opening and closing. Activation of the oocyte apparently occurs outside of the operculum. An animal removed from its tube is inefficient in transferring its eggs into the operculum; consequently, the confines of the tube are necessary. It seems reasonable to suppose that gametes are released *via* rupture of the body wall (Potswald, 1967) into the space formed by the ventral fecal pellet groove and wall of the tube. The oocytes are here fertilized and are swept, by means of ciliary action, across the achaetous zone, forward along the dorsal surface of the thorax, and finally into the center of the branchial crown which is completely withdrawn into the tube at spawning. Having reached the branchial crown, the fertilized eggs pass into the operculum by means of the opercular pore which opens to the center of the crown (Fig. 10).

Inefficiency in egg transfer in the case of adults removed from their tubes is not total. As can be seen from an examination of the data, a number of animals were successful in transferring a portion of their brood and, in fact, two were 100% effective in transferring relatively small broods. *Spirorbis morchi* has a well developed thoracic cloak which extends posteriorly and attaches ventrolaterally on about the sixth abdominal segment. The under surface of the cloak is ciliated and could conceivably simulate, to some degree, the confines of the missing tube. The latter explanation would hold true especially for animals which assume an abnormal position, *i.e.,* ventral surface of the thorax facing the substratum, in which case the cloak would serve as a natural trough into which the spawned oocytes could fall. It is common for animals removed from their tubes to assume such a position.

A major problem yet to be solved involves the controlling factors operating in opening and closing of the opercular pore. Perhaps control is endocrine in nature and correlated with spawning. At the level of light microscopy, histological investigation failed to reveal muscle fibers in the area of the pore. In an attempt to determine the possible cytological basis of contraction, a study of the opercular epithelium, at the ultrastructural level, is presently being undertaken.

Discussion

Functional hermaphroditism is found in nearly all phyla of invertebrates (Coe, 1943). Generally, when eggs and sperm develop concurrently in the same animal there are mechanisms involved which insure that self-fertilization does not take place. Nevertheless, there are documented cases of self-fertilization in various invertebrate groups. Smith (1950) has demonstrated, quite conclusively, self-fertilization in *Neanthes lighti,* an hermaphroditic, viviparous nereid which occurs along the California coast, from estuaries and sloughs of higher than normal salinity to rivers where salinity may be less than 2% that of sea water. Within the phylum Mollusca there are many instances where self-fertilization has been demonstrated, especially among the pulmonate gastropods where individuals may reproduce by self-fertilization generation after generation when experimentally isolated (see Coe, 1943, 1944 for review). Nyholm (1951) has shown that in *Labidoplax buskii* (Echinodermata, Holothuroidea) artificial self-fertilization can occur on a limited scale during the height of the breeding season. Finally, in the Arthropoda there is evidence that self-fertilization is possible in some barnacles (Barnes and Crisp, 1956; Barnes and Barnes, 1958).

In the examples cited it is obvious that eggs and sperm from the same individual are physiologically compatible; however, in most of the cases mentioned, cross-fertilization is probably the normal mode of reproduction, or at least it occurs much of the time. Whenever selfing is occasional or periodic, it need not cause any essential change in the evolutionary pattern of the species; the gene pool continues to be a reality. If, however, self-fertilization becomes obligatory rather than facultative, pure lines or clones are produced which do not exchange genes; the genotype of each line becomes a closed system capable of changing only through mutation or through reversal to biparental sexuality (Dobzhansky, 1951). In the latter case, the species, by virtue of selfing, robs itself of much evolutionary plasticity and actually gives up the benefits of sexual reproduction.

The observations presented in this report on the biology of fertilization in *Sp. morchi* are, to some extent, comparable to the observations of Gee and Williams (1965) on *Sp. borealis* and *Sp. pagenstecheri.* Although Gee and Williams did not rear animals from settlement to maturity in isolation, they did obtain data from isolated adults indicating that *Sp. borealis* and *Sp. pagenstecheri* are capable of self-fertilization. Gee and Williams make the assumption that cross-fertilization occurs when a number of worms are placed together and point out that in both species the viability of the progeny produced in association is noticeably greater in comparison with the progeny produced in isolation. This decrease in viability may be due to semi-lethal recessive genes. An apparent decrease in viability is also observed when *Sp. morchi* is allowed to reproduce in isolation.

Dasgupta and Austin (1960) report that they have evidence which suggests that the hermaphrodite serpulids *Spirorbis* and *Filograna* have been derived from triploids with a loss of a single chromosome. According to their study, *Serpula crater* has a diploid number of 14, and considering the "ancestral serpulid" as being $2n = 14$ then *Spirorbis* and *Filograna* are $2n + n - 1 = 20$. Dales (1963), presumably on the basis of the latter work, has made the interesting suggestion that self-fertilization may have arisen because of the difficulty of chromosome pairing. Studies such as Dasgupta and Austin's which suggest the evolution of a group through polyploidy are often based, as is theirs, on chromosome numbers alone without study of chromosome morphology, and, as White (1954) points out, hermaphroditic forms capable of self-fertilization do not seem to show any more polyploidy than those in which cross-fertilization is obligatory. Although, at present, there is no concrete evidence that *Spirorbis* normally cross-fertilizes, there are cogent arguments in favor of this view. One of the best arguments, aside from the genetic implications discussed above, is that offered by Gee and Williams (1965) who argue that the gregarious settling behavior exhibited by *Spirorbis* larvae (Knight-Jones, 1951) would seem to allow cross-fertilization in a population. Conceivably, an animal, just prior to spawning, could draw into its tube, by means of the respiratory current, sperm from an adjacent animal which has already spawned. It is concluded that *Spirorbis* is capable of self-fertilization and this may be of some advantage to a sedentary hermaphroditic species which has a non-planktotrophic dispersal phase; however, cross-fertilization is probably the common mode of reproduction in a natural population.

Elsler (1907) described opercular brood protection in *Sp. corrugatus* Montagu and *Sp. pusillus* de Saint Joseph as taking place between the calcareous plate and the opercular epithelium. Borg (1917) found that brood protection in *Sp. pagenstecheri* occurs in the same fashion as described by Elsler. Apparently, the structure of the operculum in the last-mentioned species must be quite different from that in *Sp. morchi* and is deserving of further study. Bergan (1953a) describes brood protection in *Sp. granulatus* as occurring in the opercular ampulla as it does in *Sp. morchi.* Bergan also observed the presence of the opercular pore; however, he was of the opinion that it was too small to allow the passage of eggs and did not attempt to determine its function. In the present study on *Sp. morchi,* it has been observed that the opercular pore is capable of opening and closing, and, in fact, is the only entrance into the opercular ampulla. The mechanisms by which the pore operates have yet to be elucidated.

Elsler (1907), Borg (1917), and Gravier (1923) all speculated that opercular brood protection is a recent development as compared with brood protection in the tube. According to Borg, brood protection in the operculum has been found only in species having three thoracic segments and never in species having 3½ or four thoracic segments. This would fall in line with the idea proposed by Caullery and Mesnil (1897) that the genus *Spirorbis* has been evolved from other Serpulidae by a gradual incorporation of thoracic segments into the achaetous region. Although a very interesting hypothesis, and one worthy of consideration, it would be of interest to know what advantage, if any, opercular brood protection has over brood protection in the tube. Perhaps a comparative physiological study of embryos developing under the two different modes of brood protection might shed some light on this problem.

I wish to thank Dr. Robert L. Fernald, Director of the Friday Harbor Laboratories, for his persistent encouragement and help throughout the course of this study. Dr. W. Siang Hsu and Dr. Paul L. Illg are thanked for their many helpful suggestions.

SUMMARY

1. Data obtained from *Spirorbis morchi* larvae reared from settlement to maturity in isolation, together with observations on isolated adults, provide evidence that *Sp. morchi* is capable of self-fertilization. Self-fertilization in *Spirorbis* is believed to be facultative and not obligatory.

2. The histology of the opercular brood pouch in *Sp. morchi* is described, and it is concluded that the only opening into the brood pouch is by means of an opercular pore.

3. Observations on the brooding behavior of *Sp. morchi* have led to the conclusion that spawning takes place when the animal is completely withdrawn into its tube.

4. An explanation as to how oocytes are deposited into the opercular brood pouch is advanced.

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TEMPERATURE RELATIONSHIPS OF POIKILOTHERMS AND THE MELTING TEMPERATURE OF MOLECULAR COLLAGEN

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Biochemical and biophysical data are beginning to accumulate for various proteins of a wide range of animals. These data include the amino acid analyses, molecular weights and dimensions of the molecular units, as well as their thermal and chemical properties. Of these, the protein of connective tissue, collagen, is probably the most widely studied from a comparative point of view (Harkness, 1961; Gross, 1963). It is the main fibrous component of the skins and tendons of the vertebrates and occurs in animals of most of the other phyla. It can be prepared in a fairly pure form from the tissues of most animals and is identified by its characteristic high angle x-ray diagram and its distinctive amino acid composition. In the native state (*e.g.*, tendon, skin, cuticle) or in its molecular state[1] in dilute solution, it exhibits fairly sharp, reproducible melting points. The melting of collagen is predominantly a first order phase transition (Garrett and Flory, 1956), and as such is virtually independent of time and takes place over a small temperature range.

These melting points, *i.e.*, the bulk melting temperature T_S and the molecular melting temperature T_D, are known to correlate with the upper limit of the environmental temperature (where these are available) of the animal concerned (Gustavson, 1956; Leach, 1957; Rigby, 1967a). Thus, all mammals with body temperatures similar to man have the same molecular melting temperature in water, $\backsim$ 36° C. (body temperature 37° C.) while the collagen of cod (a cold-water fish) melts at 15° and that of tuna fish (warm water) melts at 27° C.

For many collagens, T_S and T_D also correlate with the sum of two of the amino acid residues present in the molecule—proline and hydroxyproline (see Harrington and von Hippel, 1961). However it has recently been found (Rigby, 1967a; Fujimoto and Adams, 1964) that some earthworms and the parasitic worm *Ascaris* each contain two distinct collagens with respect to amino acid composition, particularly the sum of proline and hydroxyproline, yet T_S and T_D for each pair is identical (Rigby, 1967a).

It is perhaps advisable at this stage to describe one of these collagens as "collagenous" since it differs in some respects from the generally accepted definition of a collagen. Thus the cuticle of these worms (the collagen in question) while it shows the typical high angle x-ray diagram, and has an amino acid composition similar in pattern to vertebrate collagen, is secreted by epidermal cells rather than by fibroblasts, and does not exhibit the 600–700 Å axial repeat in the electron microscope. However from the point of view of this paper it is not crucial whether the cuticular protein is a true collagen or not, since, as we have already mentioned, it has the same values for T_S and T_D as the body collagen of the same worm (which collagen has all the accepted characteristics of collagen).

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[1] The molecular unit in vertebrate collagen is composed of three polypeptide chains, each in the form of a left-handed helix wound into a super helix about a common axis. The dimensions of this unit are *ca.* 2800 Å × 14Å, and the molecular weight is *ca.* 300,000 (see Harrington and von Hippel, 1961). The dimensions and molecular weights of invertebrate collagens appear to be different and not yet fully characterized (Maser and Rice, 1962; Josse and Harrington, 1964).

In this paper we should like to point out correlations between the melting properties of the collagens, and the behavior and physiological properties of a number of animals, particularly earthworms and parasitic worms. It is not meant to suggest that there is any causal relation between the melting of collagen and sudden changes in physiological behavior (although this is possible), but to emphasize the fact that the thermal properties of at least two proteins allow one to predict some of the temperature relations of the animal.

MATERIALS AND RESULTS

Bulk collagen was prepared from the following worms: *Ascaris lumbricoides* and *Macracanthorynchus hirudinaceus* which are parasitic worms from the small intestine of the hog, and three earthworms, *Allolobophora caliginosa, Digaster longmani* and *Pheretima megascolidioides*. *Digaster* is a giant earthworm found in the Kyogle district of New South Wales and attains a length of 5 feet or more. *Pheretima* is a common Japanese worm and was kindly supplied by Dr. K. Inukai, of the Department of Chemistry, Kyoto University, Japan.

All these worms with the exception of *Macracanthorynchus* have an outer covering, the cuticle, which is almost pure collagen (with the reservation as to definition noted above). This was used as one sample. After removal of the cuticle, collagen can be prepared from the body wall of the worms. This was the second source of collagen from each worm. In the case of *Macracanthorynchus* the body wall could easily be separated into two layers after soaking strips of wall in water overnight. The inner layer is collagen (high angle x-ray, 600–700 Å repeat and amino acid composition) (Rigby, 1967a). The method of purification of each tissue consisted in successive extraction for 24 hours in 0.1% trypsin, 10% NaCl and saturated NaH_2PO_4 solution, then washing in 0.9% saline. Full details of the preparative procedures, purification, identification and chemical composition of these samples have been given elsewhere (Rigby, 1967a).

The thermal stability of each collagen was determined by measuring the melting point of the bulk material in physiological saline, and the melting point of the bulk material in hydrochloric acid at pH 1. The melting point in saline, denoted by T_S, is commonly referred to as the shrinkage temperature. The melting point in HCl at pH 1 has been shown (Rigby, 1961, 1967b) to agree with the temperature of the helix coil transition of molecular collagen in dilute aqueous solution at neutral pH. This temperature is denoted by T_D. The principle of our method is the detection of the sudden increase in force when it melts, in a sample which is prevented from changing its dimensions. In saline it is crystalline aggregates of molecular units which melt, while in acid solution, swelling of the structure and subsequent dispersion of molecular units allows single molecules or small groups to melt.

The results relevant to this paper are given in Table I. The temperatures are the means of at least four determinations; the error is ±1° C.

At this point it is worth noting that for a number of soft collagenous tissues T_S and T_D are independent of the degree of purification. Thus, in the present case, strips of fresh earthworm body wall (*i.e.*, with cuticle removed) give the same values for T_S and T_D as does the collagen extracted from same by the methods described above. While it does not follow that only the collagen is melting it indicates that in soft tissue, at least, some of the physical properties of collagen are unaffected by its association with other components. This point is of particular physiological interest as the following discussion attempts to show.

The question as to the correlation of T_S and T_D with amino acid composition—in particular, with the total pyrrolidine content—is not considered to be relevant to this discussion, but it has been dealt with fully elsewhere (Rigby, 1967a).

TABLE I

Melting temperatures in physiological saline (°C.) of the collagen of various worms. T_S is the melting point of bulk, native sample, T_D the melting point of molecular unit. B, body. C, cuticle. These transitions were measured on the bulk native material (Rigby, 1961, 1967b)

	P. mega-scolidioides	*Digaster longmani*		*A. caliginosa*		*As. lumbricoides*		*Macracan-thorynchus*
	C	C	B	C	B	C	B	B
T_S	37	34	40	34	40	56	59	62
T_D	22	22	22	22	22	40	38	42

DISCUSSION

The first thing to be noticed from Table I is that all the earthworms have the same value of T_D for both cuticle and body collagen. Also, the hog parasitic worms and hog intestinal wall collagens all have similar values of T_D. The values of T_S tend to be the same for each group as well, although a number of factors affect T_S without affecting T_D. In any case it is of more fundamental interest to consider T_D. It will be seen that T_D for the parasitic worms is close to that of their environmental temperature, namely that of the hog. This does not appear to be a coincidence, for it is well known that the T_D values of the collagens of the mammals, man, rat, sheep, and ox, are all virtually the same as that of their deep body temperature, as was mentioned in the introduction. This information is summarized in Figure 1 for a wide range of animals and environments. The simplest explanation of this correlation is of course, natural selection, although why the two temperatures should almost coincide is a question which requires further study. It might be thought, for example, that T_D would be well above the maximum environmental temperature.

The second point of interest in Table I is the value of 22° C. for T_D of all the earthworm collagens. According to Figure 1 this would imply an upper limit of about 22° C. for the environmental temperature of these worms in their natural habitat. There are two kinds of evidence in the literature which are

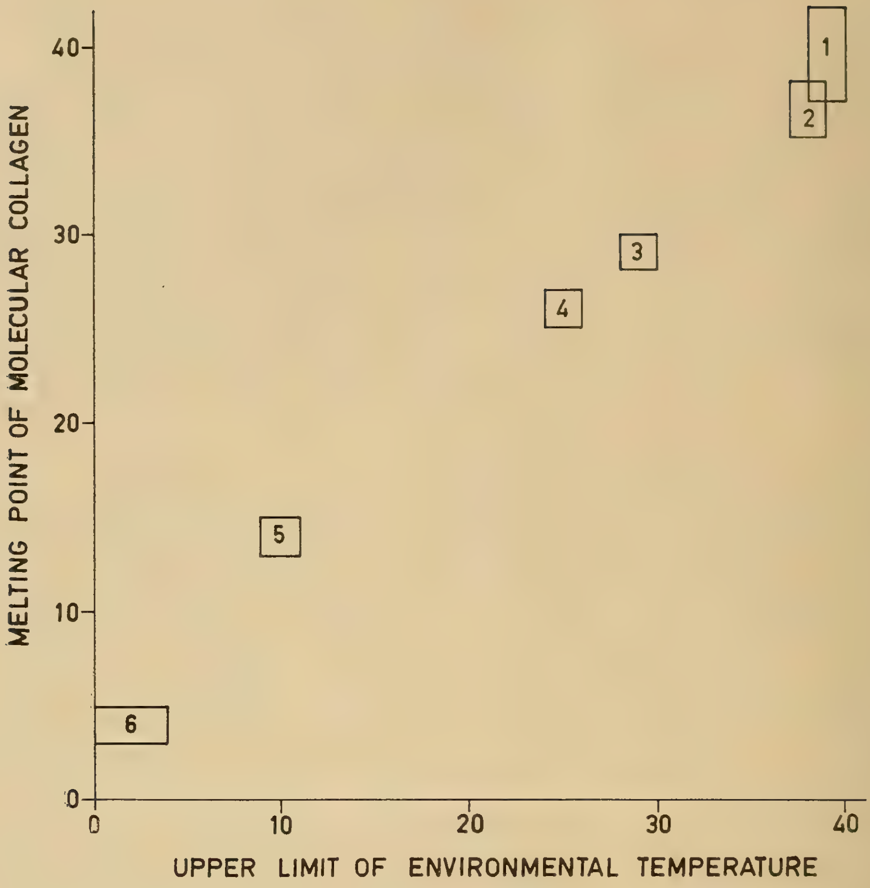

FIGURE 1. The relation between the melting point (T_D), of the collagen molecule from various animals, and the approximate temperature of the upper limit of their environment. 1. *Macracanthorynchus, Ascaris* and hog; 2. rat, human, cow; 3. snail (Newell, 1966) (*Helix aspersa*); 4. tuna skin (Dakin, 1953); 5. cod skin (Tait, 1952); 6. Antarctic "ice-fish." The reference numbers are for environmental temperatures. The Antarctic "ice-fish" is the subject of a detailed paper in preparation.

consistent with this view. These are (i) the thermal preference range of the worm and (ii) the relation between the environmental temperature and the body temperature of the worm. We have only been able to examine collagens of two of the worms studied by others: viz: *Pheretima megascolidioides* and *Allolobophora caliginosa.* In both cases we must assume that temperature was the only factor under observation and secondly, point out that our *Allolobophora* were of Australian origin while the published work to be discussed was performed with North American and Egyptian animals.

We turn first to studies of temperature preference ranges and consider *A. caliginosa* particularly, since we have data for the collagen of this animal. Grant (1955) and El-Duweini and Ghabbour (1965) have studied the thermal preference ranges of this worm, as well as *E. foetida* and *P. hupeiensis* and *P. californica* and *Alma* sp., respectively. Although the lower limit of this range varies, Grant (1955) quotes 23° C. as the upper limit of each animal. The results of El-Duweini and Ghabbour (1965) are more difficult to interpret since they used *A. caliginosa* from two regions of Egypt. Both groups of worms showed a wide temperature preference range but in both cases there was a sharp drop in the number of worms preferring temperatures above 28° C., this number was about 10% of the total. For *Alma* sp. 88% of the population preferred the range 23–28° C. with the peak at 25° C. *Ph. californica* were almost equally distributed between 26° C. and 35° C. Grant (1955) allowed 24 hours before deciding on a worm's temperature preference, whereas El-Duweini and Ghabbour (1965) allowed 0.5 hour and it could be questioned whether theirs are equilibrium results. However for *A. caliginosa* a T_D value of 22° C. appears to be correlated with the temperature of the upper limit.

Considering next body temperature, we draw attention to the work of Kim (1930) who studied *Pheretima megascolidioides*. His main conclusion was that between 6° and 23° C. the body temperature of *Pheretima* was the same as that of the surrounding medium, but beyond 24° C., for unknown reasons, its body temperature varied erratically. His results imply an upper limit of temperature preference of 23–24° C., and this again (as with *A. caliginosa*) correlates with T_D.

Further, on the subject of sudden physiological changes due to temperature increase such as the one just described, it is relevant to mention the results of Hatai (1922) concerning muscular contraction in several Japanese earthworms. He found rhythmic contraction to be normal and unaffected by temperature up to 23° C., beyond which the form of contraction altered suddenly.

The preceding results and discussion show that earthworms from a number of species and habitats have collagens with identical thermal stability, and, in turn, similar upper limits for their temperature preference range. This value, 22° C., may be characteristic of the family.

It is now of interest to examine the so-called upper lethal temperature. The definition of this temperature is arbitrary and values quoted for a given animal are thus widely spread. Assuming as before that the upper lethal temperature depends only upon temperature and that other factors, such as water content, are kept constant it would seem that the upper lethal temperature could be defined by extrapolation of a time-temperature relation from high temperatures until time and temperature become independent. In other words, above the true lethal temperature, time and temperature would be approximately inversely proportional; below, no relation would be evident.

In the literature on lethal points, El-Duweini and Ghabbour (1965) quote 39° C. for *A. caliginosa* with similar figures for *Ph. californica* and *Alma* sp. Their definition was the temperature at which more than half the sample dies after an exposure of 0.5 hour. Wolf (1938) and Hogben and Kirk (1944), using *L. terrestris* and times of 400 minutes and 12 hours, respectively, gave lethal temperatures of about 29° C. Grant (1955) using an interpolation method and exposure times up to 48 hours gives values of 26° C., 25° C. and 23° C. for *A. caliginosa*, *P. hupeiensis* and *E. foetida*, respectively.

It would appear that the high values quoted by El-Duweini and Ghabbour

(1965) are due to the short exposure time and the inverse relation between time and temperature discussed above, although a factor could be that the worms have some measure of acclimatization to the hot Egyptian climate as mentioned by these authors. Of greater significance, however, is the fact shown by Table I that the thermal shrinkage of earthworm bulk collagen takes place between 35 and 40° C. The values of Grant (1955) are quite close to the upper limit of the preferred temperature range and this is consistent with the data of Kim (1930) where it was seen that above this upper limit definite physiological irregularities become apparent. In other words irreversible changes have begun to occur in the animals' tissues, but long times may still be required before death occurs. One further point which should be mentioned here is that whereas any molecular melting which takes place in bulk collagen is reversible as long as there is no mechanical stress present and T_D is not exceeded by 5–10° C., the melting which occurs at T_S is irreversible except, possibly, in the case of cross-linked collagens such as elastoidin. Thus, so far as collagen is concerned the temperature could exceed T_D for short periods of time without necessarily affecting the animal adversely.

Summarizing, we can say that for the earthworms discussed, the melting point of the collagen molecule (the basic unit of their connective tissue) is very close to the value of the upper limit of their preferred temperature range and in turn to the upper lethal temperature. They are similar to mammals in this respect and to parasitic worms of mammals which at some stage of their life cycle exist at much lower temperatures. Whether or not collagen is uniquely involved in determining these limits cannot be said at this stage, although the fact that it is a protein with a very low rate of turnover (with certain exceptions, Harkness, 1961) and sharp melting point is perhaps significant. It should be pointed out, however, that it is likely that all the animal proteins begin to denature at temperatures above T_D. Finally it has recently been shown by Newell (1966) that the oxygen uptake of various poikilotherms increases abruptly at temperatures which correspond with the upper limit of the environmental temperature, and in one case for which collagen has been isolated (*Helix aspersa*), to the T_D for this collagen, *viz.* 27° C. (Rigby 1967b).

SUMMARY

The molecular unit of collagen, the main fibrous protein of connective tissue, undergoes a first order phase transition at a characteristic temperature, when heated in physiological saline. For the collagen of mammals this temperature is known to almost coincide with the deep body temperature of the animal, and for a number of poikilotherms, with the upper limit of their environmental temperature. We have measured the transition temperature of the collagens of a number of worms and now report that:

1. The molecular unit of the collagen of two hog intestinal worms undergoes this transition at temperatures close to that of the body of their host.

2. For a number of earthworms for which there are precise data on thermal preference limits, the molecular unit of the collagen of these animals has a transition temperature which is very close to the upper limit of the thermal preference zone.

3. There is no evidence of any causal relation between the transition temperature of molecular collagen, and the abrupt physiological and behavioral changes which become apparent at the same temperature. However, the results are direct evidence of a specific protein having easily measurable thermal properties which reflect thermal properties of the whole animal.

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MIGRATORY ORIENTATION OF AMBYSTOMA MACULATUM: MOVEMENTS NEAR BREEDING PONDS AND DISPLACEMENTS OF MIGRATING INDIVIDUALS

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Migrating spotted salamanders, *Ambystoma maculatum,* tend to enter and leave breeding ponds near the same point (Shoop, 1965). This paper confirms the original observation and presents results of observations and experiments designed to further elucidate the problems of migratory orientation of spotted salamanders.

METHODS

Animals from two breeding ponds (A and B) in Wellesley, Massachusetts were studied from 1964 to 1967. Nylon-coated, screen fences 40 cm. high, buried 15 cm. in the soil, surrounded the ponds; deep can traps were buried at intervals along both sides of the fences. Other fences from 10 to 200 m. long with traps along both sides were located at varying distances (15 to 175 m.) from the fence surrounding Pond A. Details concerning Pond A and methods are described by Shoop (1965); Pond B is in mixed hardwood-pine forest adjacent to a golf course.

Traps were checked regularly throughout the day and night during the breeding season and at dawn during the rest of the year except when snow covered the traps. The trapping method exposed the animals to increased predation by raccoons; several salamanders were killed before a wire with electrical charger was added around the outer face of the fencing about 15 cm. above the ground. Some animals were released on the Wellesley College campus in a 40-m.-diameter arena with traps along the inner face of a 0.5-m.-high surrounding fence. All animals were individually marked by toe-clipping. Statistical procedures follow Batschelet (1965).

CONFIRMATION OF EARLIER OBSERVATIONS

The relation between point of entry and exit of Pond A by animals during 1964, 1965, and 1966 and mean vector values, 99% confidence intervals for mean vectors, and angular deviations are presented in Figure 1. In all cases corrections for trap intervals are included in the vector determination. The r values vary from 0.42 to 0.55, indicating some dispersion but non-random distribution. In all three cases the mean exit vector falls within the consistent direction (Shoop, 1965) in relation to the point of entry into the pond, and the 99% confidence intervals for the mean vectors overlap. The data for the three years are not

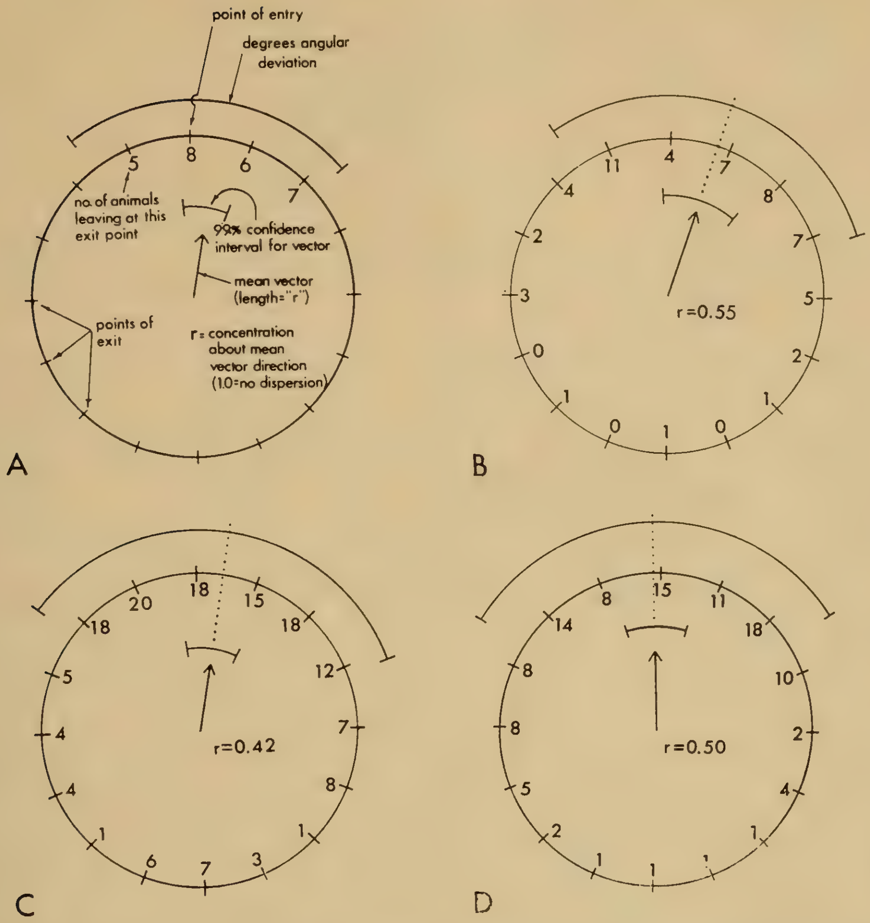

FIGURE 1. Relation of exit point at breeding pond border to point of entrance (top of circle is point of entry, not necessarily north): A, explanation; B, 1964; C, 1965; D, 1966. The 99% confidence intervals of the mean vectors overlap indicating highly significant correlation between years.

significantly different. Animals collected only at entry or exit, or animals used for displacement studies in 1966 were not included in the vector determination.

When interpreting the data of movements into and out of the breeding pond, certain aspects of the experimental design should be considered. The pond was circular with a steep bank, a rather uncommon type of vernal pond. This allowed for close placement of the surrounding fence with traps at the sixteen compass points, and since all recorded directions are in relation to the center of the pond, the data may be more refined than is biologically significant. In reality, a migrating salamander may not move toward the center of the pond but merely attempt to intercept the border. An animal moving from a point east (90°) of the pond center may intercept the border anywhere from 0° to 180° and therefore have a

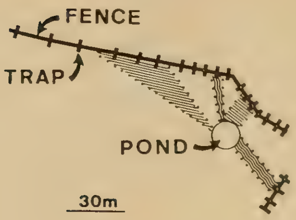

FIGURE 2. Corridors of travel of 4 animals (1967) selected to avoid overlap and show range of variability (N = 36). One-half distance to next trap included in corridors. The fences and traps border forest areas.

"successful" migration. Conversely, the animals may leave the pond anywhere between 0° and 180° and be on the east side of the pond. Considering the amount of dispersion, this type of orientation may actually be more typical of the biologic situation. That a statistically significant number of animals did choose consistent headings in relation to the point they entered the pond indicates that the animals tend to use the same track even though their terrestrial retreats may not be closest to the entry point. Additionally, animals determined as taking "poor" headings may correct for their "error" after leaving the pond.

Studies utilizing fences at varying distances from breeding ponds in 1966 and 1967 seem to confirm the above suppositions. When migrating in a meadow to and from the pond many animals tend to limit their movements when within 100 m. of the pond to a narrow corridor 10 to 30 m. wide. Some corridors used by the salamanders in 1967 are represented in Figure 2. In 1967 36 animals were caught at the forest border and pond border at entry and exit (4 captures each). The corridor used in crossing the meadow to and from the pond may have little relation to the movement of an animal in the forest. Figure 3 shows capture points of an animal moving in an easterly-westerly corridor at a great angle to a line drawn between capture points at the forest border and in the forest. Time between the last two captures was 6 days.

Of the animals collected in successive years, most seem to travel in the same corridor to and from the pond (Fig. 4), although some apparently use different corridors. A pattern of different corridors for entry and exit (seen in 3 animals) may be repated in successive years (Fig. 5A). A few animals seem to move in no definite pattern (Fig. 5B).

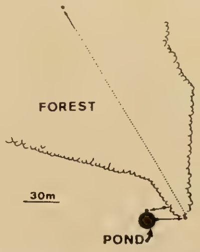

FIGURE 3. Movements of a single individual, 1967. ● = capture point; → = direction of movement; . . . . = shortest distance between capture points.

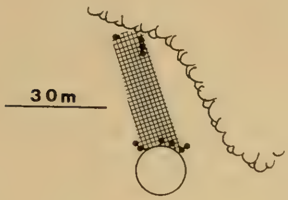

FIGURE 4. Capture points of a single individual, 1965–67. Dots around pond represent captures on entry and exit, 1965–67. Dots at forest border, entry and exit points 1966–67. Shaded area is probable corridor of travel.

EXPERIMENTS INVOLVING DISPLACEMENT OF MIGRATING SALAMANDERS

Displacements in the field

Since migrating animals tend to use the same track into and out of a breeding pond, they may possess an ability to utilize a directional tendency (Griffin, 1952, "Type II orientation," or Schmidt-Koenig, 1965, "compass orientation"); if familiar landmarks or local sensory cues are unavailable or not utilized, they may move in a prescribed direction or its reciprocal (along a directional axis). Assuming that only a compass sense is involved, the animals should fail to reach the pond in the following situation: animals are captured at the pond border, carried 90° around the pond, and then taken in a straight line away from the pond and released at a location where familiar landmarks are obscured. For example, if an animal that had migrated from a terrestrial retreat east of the pond to the east border of the pond, was moved to a point north of the pond, it would travel east or west and miss the pond. The following experiments tested this possibility.

Twelve animals captured at night in the ENE trap at Pond A border were placed in opaque containers and transported at dawn to four locations. Three animals were released 20 m. from ENE; three at 135 m. from ENE; three at 45 m. from NNW; and three at 175 m. from NNW. Of six animals collected in trap NNW, three were released 20 m. from ENE and three 45 m. from NNW. Results of this experiment are presented in Table I. One animal released 135 m. from ENE was killed by raccoons on return to the pond fence.

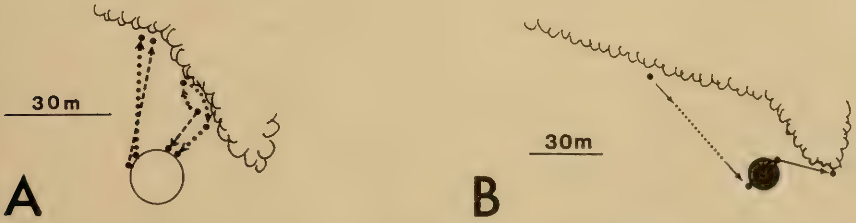

FIGURE 5. A, Movements of a single individual, 1966 (dashed line), 1967 (dotted line). Looping movements (1967) due to dry weather with animal returning to forest. Note different exit and entry corridors. B, Movements of an individual showing no regular pattern, 1967.

One of three males caught at the pond border in trap NNW and transported to a point in a meadow 45 m. NNW of this trap behaved unexpectedly when released under cloudy skies and a NW wind (*ca.* 10 knots). Instead of crawling under cover as animals released in daylight usually do, the salamander, out of sight of the pond or its bordering trees, extended its limbs and slowly moved its head from side to side. Within a few seconds it began to move uphill at a heading of 153°. After moving approximately 3 m. in a straight line, it stopped, repeated the limb extension and lateral head movements, changed its heading to 170° and continued this heading with occasional pauses, moving over the ridge and down to trees bordering the pond where it burrowed under leaves west of the pond center. Slight detours around obstructions were made, but the resulting path of travel on the 34 m., 170° route varied less than 1 m. from a straight line. This animal paused nine times during the 27-minute excursion with a rate of travel during the active portions of about 2 m. per minute.

Except for animals released 20 m. from ENE, all the returning animals had to move some distance uphill before reaching the pond. Animals released 175 m. from NNW were in a different drainage system and had to cross a 9-m.-high ridge with 35° slopes. The time required to return to the pond varied from two to ten days but extremely dry weather also slowed normal migration of other animals at this time. One of the two animals not reaching the pond returned the following year.

TABLE I

Results of releases of animals originally captured at pond border

Point of capture at pond	Point and direction of release	Returns to pond/ no. released
ENE	20 m. ENE	3/3
ENE	45 m. NNW	3/3
ENE	135 m. ENE	2/3
ENE	175 m. NNW	3/3
NNW	20 m. ENE	2/3
NNW	45 m. NNW	3/3

In a related experiment late in the 1966 breeding season, 57 animals moving from many directions and collected on arrival at another pond (B) were released at points 200 and 250 m. from Pond B. A salamander returning from the 200 m. release point could encounter any of three other ponds never known to harbor breeding salamanders; return from the 250 m. release point involved crossing a lawn, a large, steep (35° slope) 25-m.-high ridge, and possibly encountering a permanent pond. Two days after the releases the area between the pond and the release points burned, adding strong odors to the area.

Of 28 animals released 200 m. from the pond, 13 were eventually recaptured at the pond border, 7 in the same season, 6 more one year later. Nine of 29 released at 250 m. were eventually recaptured at the pond border, one the same season, one in the autumn, and 7 the following spring. Returns of the same season tended to enter the pond at points nearest the release point. In both cases returns after two years approximated the percentages of returns by non-displaced animals.

Since the ability of some animals to return from distances up to 250 m. was established, four animals (two males, two females) were collected at dawn at Pond B border early in the 1967 breeding season, placed in covered containers and carried in a truck for one day. At night, phosphorescent vinyl bands were placed around the bodies of the animals, and they were released on a golf course fairway during a heavy rain, 500 m. from Pond B. Return to the pond involved crossing over 100 m. of lawn, encountering up to four temporary ponds, one road, and a small stream.

At release all four animals took headings within 15° of the pond direction. They were turned around by hand but they immediately headed in the pond direction (uphill) and moved approximately 25 m. before being lost from view. After 11 days one of the animals (♀) arrived at the pond border trap nearest the release site. The other three animals were not seen again.

If orientation to a breeding pond is a simple geotaxis, rheotaxis or random search, some migrating animals collected while moving to a breeding pond and displaced close to another breeding pond should then move into the "new" pond. Negative results would suggest that geotaxis, rheotaxis, or random search must be coupled with unknown qualitative factors of the pond itself or that these taxes are not the major factors used in finding the pond.

To test for the above taxes, nine salamanders captured as they moved to Pond B were carried at night in covered containers to sites near Pond A. Three animals were released in a short-grass meadow at each of three compass points (0°, 90°, and 270°) 10 m. from the Pond A border. In two cases, the pond was downhill from the release site; in the other, slightly uphill. None of the animals (6 males, 3 females) moved to the new pond (A). Eight of nine control animals (captured at the Pond A border) moved to Pond A. The following year three of the displaced animals (all males) were recaptured at Pond A, and two of these animals were seen the succeeding year.

Six males transported from the Pond B border and released in the center of Pond A stayed within Pond A from four to 22 days. Their exit directions bore no relationship to the directions from which they attempted to enter their original pond (B) or the direction to Pond B. One of these animals returned to Pond A the following year and was captured while migrating in a nearby wooded area the succeeding year but not at the Pond A border.

Displacements to an arena

In an effort to remove all possible local cues surrounding breeding Pond B, 106 migrating animals were transported to the laboratory and maintained in running lake water aquaria for periods varying from three to eight days. These animals were released one or more times in a 40-m.-diameter arena on the Wellesley College campus under cloudy and clear skies at night during the normal migration period. No significant differences in performances between males and females or between cloudy and clear conditions were noted. A total of 547 releases was made, and the capture points around the inner face of the fence were determined.

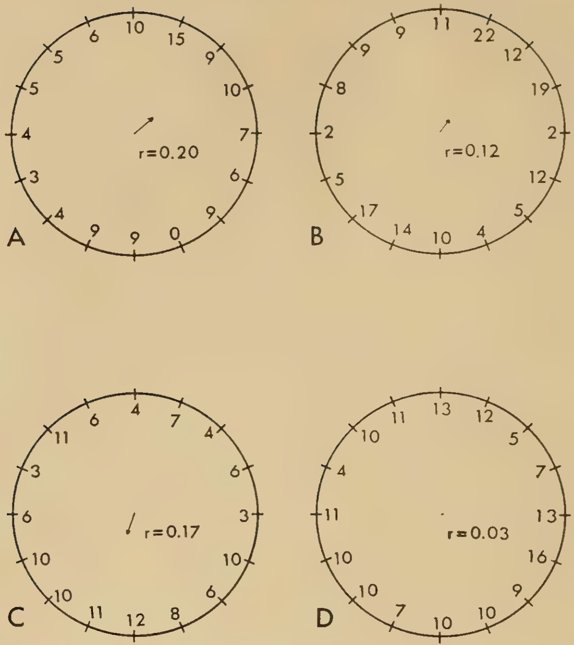

FIGURE 6. Representation of capture points of animals released in arena about one mile from home pond. A, Relation of headings of animals released facing 90° to the left of their heading when captured in the field; top of circle represents original heading in field, not north. B, Headings of animals released by removal of covering container, in relation to original heading in field; top of circle represents original heading. C, Compass headings of animals released facing ENE; top of circle is N, SSE is direction to home. D, Compass headings of animals released by removal of covering container; top of circle is N, SSE is direction to home.

The data obtained by releasing animals facing 90° to the left of their original heading in the field, or released at chance headings (by removing a covering container), in relation to direction of original field heading are random ($r \leqq 0.20$; Fig. 6 A, B). Pooled results of releases of animals faced ENE or released by removal of a covering container (Fig. 6C, D, respectively) are random ($r < 0.20$) and show no relation to the true homeward direction, SSE. These results demonstrate that under the conditions of these experiments the animals have no preferred direction in relation to the way they were traveling when collected or in relation to the direction of the home pond approximately one mile away.

DISCUSSION

Whitford and Vinegar (1966) displaced *Ambystoma maculatum* adults up to 420 ft. (*ca.* 128 m.) from a breeding pond and found they were able to return to the pond, apparently aided by a rheotactic, or perhaps olfactory, response, thereby adding support to a finding of rheotactic orientation of *A. maculatum* by Finneran (1951). Under the conditions of my experiments rheotaxis apparently plays little or no role in locating the home breeding pond.

If the four animals displaced 500 m. from their home pond had no sensory contact with that pond or known routes of travel to it, they may have used a true navigation ability (Type III orientation of Griffin, 1952; reverse displacement or bicoordinate navigation of Schmidt-Koenig, 1965) in their initial oriented headings. The life-time movements of spotted salamanders are unknown; therefore displacements to unfamiliar territory are contingent upon thorough studies before true navigation can be established.

The random headings of animals released in the arena about 1 mile away from the home pond may parallel homing studies with *Taricha rivularis* in California (Twitty, 1959, 1961). Oldham (1967) reported similar results in displacements of green frogs, *Rana clamitans.* David L. Grant (personal communication), who is continuing the studies of *Taricha* orientation, utilizes a much larger "arena" to determine headings of animals displaced large distances. Apparently *Taricha* requires some time and space to determine and maintain correct headings to the home area, but in a relatively small arena *Taricha granulosa* can utilize a sun compass to move on land in a direction that would take the animals to a home shore (Landreth and Ferguson, 1967).

Laboratory conditions may affect the performances of *A. maculatum* released in an outdoor arena. Since handling and laboratory storage of females may prevent ovulation in some unknown way (Shoop, 1967), a physiologic barrier resulting from these conditions has been demonstrated. Perhaps a similar "upset" was responsible for the results of the arena release experiments.

Since spotted salamanders leaving a breeding pond often maintain a very similar or identical track away from the pond as they took to it, the animals could conceivably run through an imprinted cue system obtained as they left the ponds as juveniles and reverse it on returns to the pond, although day-length, temperature, sun altitude, and star patterns are different. Whether they always migrate every year only in familiar territory (home range) remains unknown. Uzzell (personal communication) and I have observed other ambystomatids migrating to areas where temporary ponds were not yet formed, as is often the case with the terrestrial egg-layer of the group, *A. opacum.* I observed a population of *Ambystoma talpoideum* moving into an area where a breeding pond was previously located but road construction between breeding seasons had obliterated the previous habitat. Heusser (1960) made a similar observation of migrating toads (*Bufo bufo*). While local visual or olfactory cues may play important roles in orienting to a breeding pond, in these instances, other cues were probably operative.

Many salamanders show a decided preference for a breeding pond and return year after year. Others may adopt a new breeding pond if displaced. Until lifelong records of movements or home ranges and perceptive abilities of amphibians are known, definitive statements regarding navigation and sensory mechanisms utilized in orientation will be open to question.

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SUMMARY

1. Migrating spotted salamanders entered and departed from breeding ponds at or near the same point. This pattern was repeated for three consecutive years.

2. Animals migrating in a meadow to and from the breeding pond often traveled in a corridor 10 to 30 m. wide. Several variations from this plan were noted.

3. Spotted salamanders displaced up to 500 m. may return to the home breeding pond.

4. Migrating adults transferred from the border of one breeding pond to within 10 m. of another breeding pond did not move to the new pond as did controls.

5. Migrating adults transported from the border of one pond to the center of another failed to orient to the old pond or in the direction they were moving when captured.

6. Some adults adopted a new breeding pond when displaced.

7. Animals moved to a laboratory and then released outdoors in an unfamiliar area failed to orient to their pond or in the direction they were moving when originally collected. Handling and laboratory conditions may have altered normal behavior.

8. Since lifelong records of salamander movements are lacking, certainty of navigation ability may be questioned.

9. Sensory bases for orientation remain poorly known.

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POLAROTAXIS IN COPEPODS. I. AN ENDOGENOUS RHYTHM IN POLAROTAXIS IN CYCLOPS VERNALIS AND ITS RELATION TO VERTICAL MIGRATION

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In many terrestrial arthropods, the ability to detect polarized light is used to find geographical directions by means of sky polarization patterns which change with the sun's position in the sky (von Frisch, 1948; Pardi and Papi, 1953a, 1953b; Birukow, 1954; Vowles, 1955; Wellington, 1955; Papi, 1955). Such direction-finding is actually a special extension of the sun compass and requires that the organism possess a physiological clock to correct for the sun's movement through the sky and the concomitant changes in sky polarization.

The functional significance of polarized light sensitivity for planktonic animals has yet to be demonstrated. However, many such organisms can perceive polarized light (Baylor and Smith, 1953; Jander and Waterman, 1960) and polarization patterns are indeed present in the aquatic habitat (Waterman, 1954; Waterman and Westell, 1956; Ivanoff and Waterman, 1958; Ivanoff, Jerlov and Waterman, 1961). It is reasonable to speculate that these aquatic organisms use the polarization pattern of their environment for orientation just as do their terrestrial counterparts. This paper presents evidence that the natural pattern of polarization in the aquatic habitat is used as a frame of reference for swimming orientation in the copepod *Cyclops vernalis* and that the orientation of this animal to the plane of polarization changes rhythmically throughout the day in an ecologically meaningful way.

GENERAL BEHAVIOR

Material and Methods

As experimental animals, the fresh-water copepod *Cyclops vernalis* Fischer, collected in Clark's Pond near New Haven, Connecticut, was cultured at 20° C. in an aerated five-gallon aquarium in a darkroom having a 12-hour light, 12-hour dark cycle of illumination. Animals had lived in this culture for more than a month before experiments were begun.

To measure phototactic responses, 20 copepods were placed in a rectangular, clear Plexiglas vessel. One-half of this vessel was lined and covered with black Plexiglas to shield this half of the container from the overhead tungsten filament light source. An intensity of light set at 1100 lux was chosen to be the value incident at the water surface since this is a daylight intensity level commonly found in natural waters (Harris and Wolfe, 1955). The phototactic sign of the copepods was determined by measuring their preference for the light or dark half of the vessel at various times of day. The number of copepods in the light half of the vessel was recorded every 20 seconds over a period of 10 minutes, permitting an average to be calculated. These observations were made at 2-hour intervals over the course of the 12-hour light period.

To ascertain geotactic responses, 20 copepods were placed in a 500-ml. graduated cylinder in complete darkness; there was no light-dark cycle. The copepods were observed with a red light at all times of the day to determine their distribution in the vessel. The number of copepods in the top third, middle third and bottom third of the vessel was recorded at 20-second intervals for 10 minutes. These observations were made at 2-hour intervals during the course of a 24-hour day.

Results

The copepods were photonegative at all times during the 12-hour light period, spending 90% of the time in the dark. When an occasional animal would venture into the lighted half of the vessel, it remained at the bottom and seldom swam upwards toward the light. There was no evidence for changes, rhythmic or otherwise, in phototactic sign.

The copepods were also geopositive at all times of day, spending 85% of the time on the bottom of the graduated cylinder. Again, there was no evidence for changes in geotactic sign at any time of the day.

It was also observed that when swimming horizontally in the water, *Cyclops vernalis* directed its ventral surface upward toward the overhead light. If light was shined from below, the ventral surface then faced downward, still toward the light. This behavior indicated that a ventral light reflex was present. When swimming vertically upward, the anterior end always led with the long axis of the body vertical. When the copepod moved downward, it either actively swam with its anterior end downward and long axis of the body vertical or it passively sank with its body axis horizontal.

Cyclops vernalis tended to cling to the sidewalls and bottom of any experimental vessel and often adhered to the water-air interface when one was present. It sporadically swam freely in the water. This behavior in the laboratory agrees with the field behavior as reported by Pennak (1966). Thus *Cyclops vernalis* is essentially restricted to vegetation in the littoral zone of lakes and ponds, being seldom found in the open water. It is a browsing species that often leaves the substrate to cruise about in spaces among the vegetation. This suggests that the copepods in the laboratory culture were in normal physiological condition.

POLAROTACTIC BEHAVIOR

Methods

The apparatus used to measure polarotaxis was similar to that employed by Bainbridge and Waterman (1957). A shallow cylindrical Plexiglas cell, 26 mm. deep and 108 mm. in diameter, containing 20 copepods, was illuminated from above by a vertical beam of linearly polarized light whose plane of polarization could be altered at will. The light originated from a tungsten filament lamp and passed successively through a focusing lens system, a heat filter, a depolarizer, a rotatable Polaroid linear polarizer, the experimental cell and finally through two lenses which produced an image of the copepods on a sheet of paper on the bench beneath.

The intensity of light was 1100 lux at the upper surface of the experimental cell.

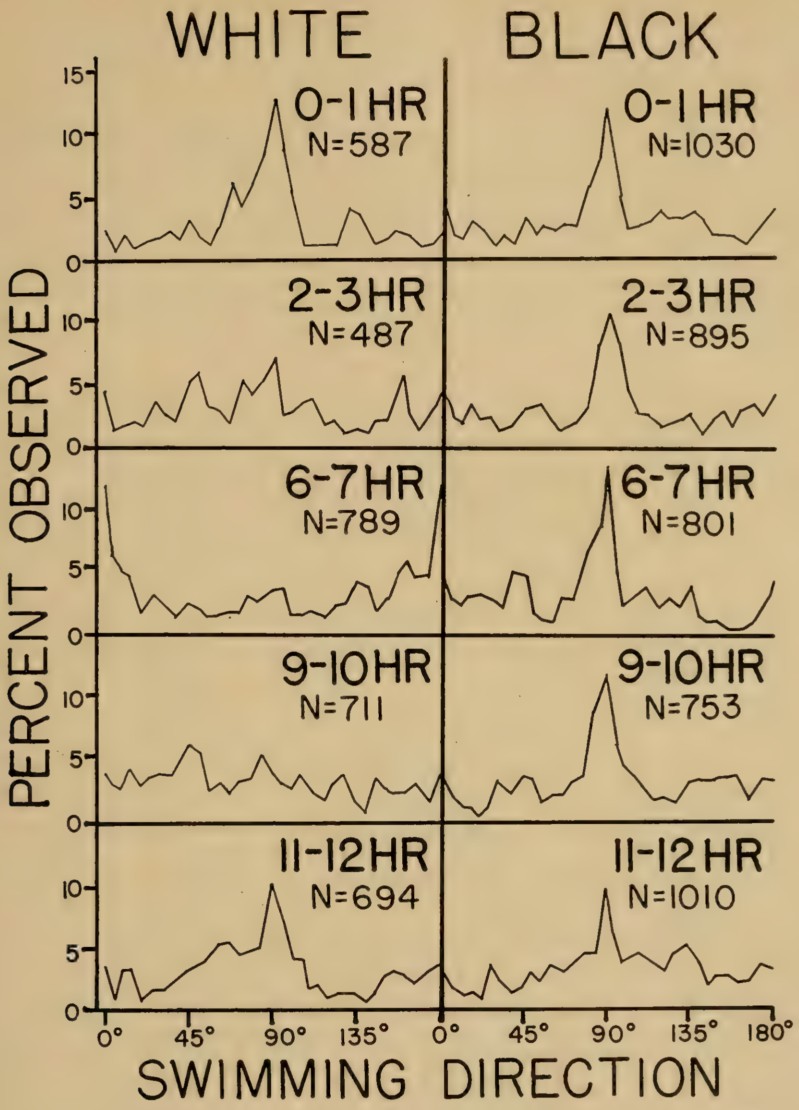

FIGURE 1. Polarotaxis at different times during a 12-hour light cycle. Abscissa gives the angle between swimming path and plane of polarization. Ordinate gives the percentage of total swimming attempts. N gives the total number of swimming attempts. WHITE and BLACK denote the color of the sidewalls and diaphragm of experimental cell.

The vertical outer walls of the cell were covered with either black or white construction paper to minimize direct reflections from the walls. In addition, the walls were shaded from direct light with a circular black or white diaphragm having an aperture 20 mm. less than the inner diameter of the cell. The meniscus at the margin of the water surface was eliminated by pressing a flat glass plate against the upper edge of the cell walls, thus sealing off the completely full vessel without an air-water interface. The water was filtered twice to reduce the differential scattering of polarized light by a turbid medium (Bainbridge and Waterman, 1958; Waterman, 1960).

Using this apparatus, the paths of the swimming copepods could be traced by following their projected images with a pencil on a piece of paper on the bench beneath the experimental cell. The plane of polarization was first set to one of four positions and noted on the paper along with the tracings. After a number of observations had been made, the plane of polarization was then changed at random to a new position and more tracings were made. A total of four different *e*-vector positions was used for each trial to eliminate the effect of any intensity or spatial cues in the cell.

The angle between the path of the copepod and the plane of polarization was then measured with a protractor at 1-cm. intervals along the length of each path tracing. The frequency distribution of the resulting angles indicates any relationship between the course of the copepods and the plane of polarization. Thus, if a copepod tended to swim perpendicular to the plane of polarization, a peak in the orientation readings would appear at 90° on the plot. The frequency distributions can be statistically evaluated as either random or with significant peaks by a chi-square test (Bainbridge and Waterman, 1957).

As indicated previously, *Cyclops vernalis* tended to cling to the bottom and sides of the experimental cell and only sporadically swam freely in the water. In order to get a sufficient number of tracings in a 30-minute period, 20 copepods were placed in the cell to insure that one or two individuals would always be swimming. The paths of the copepods were recorded at 2-hour intervals throughout the 12-hour light period; no readings were attempted during the 12-hour dark period of the illumination cycle. In the following discussion, therefore, a time of 0–1 hours indicates the first hour after the light went on (experimental "dawn") and a time of 11–12 hours indicates the last hour before the light went off (experimental "dusk"). During each 1-hour period of observation, both black and white sidewalls and diaphragm were used to discover if any light contrast reactions were present as reported in several animals by Jander and Waterman (1960).

Results

When black sidewalls and diaphragm were used, *Cyclops vernalis* oriented at right angles to the plane of polarization at all times during the 12-hour light period. However, when white sidewalls and diaphragm were used, the same copepods in the same vessel at the same times of day now showed a regular 12-hour rhythm in polarized light orientation. Under these conditions, there was a 90° orientation to the plane of polarization at the beginning and end of the light period and a 0° orientation midway through the light period. These orientation peaks were statistically significant at the 0.5% level using a chi-square test. At intermediate times, however, orientation was poor and not significant statistically, appearing to be transitional between 90° and 0° orientation. The results for one such day are shown in Figure 1. The same results, using other groups of 20 copepods, were obtained on two other days when the experiment was repeated.

POLAROTACTIC BEHAVIOR UNDER SIMULATED NATURAL CONDITIONS

Introduction

In natural waters, the angle between the plane of polarization and the horizontal depends upon the line of sight of the observer, the position of the sun in the sky and the depth of the observer in the water (Waterman, 1954; Waterman and Westell, 1956). The plane of underwater polarization is always horizontal in horizontal lines of sight toward and away from the sun's bearing, but is tilted toward the sun in all other azimuths. The amount of tilting between the plane of polarization and the horizontal is maximal at right angles to the sun's bearing and is approximately equal to the sunlight's angle of refraction at the surface of the water. However, there is a decrease in the tilting of the polarization plane as depth increases.

At sunrise and sunset, when the sun is near the horizon, the angle of the plane of polarization may be as great as 45° along horizontal lines of sight perpendicular to the sun's bearing. At midday, when the sun is nearest the zenith, the plane of polarization along a line of sight perpendicular to the sun's bearing reaches its minimal tilt from the horizontal. In fact, when the sun is in the zenith, the *e*-vector is horizontal in all lines of sight. This then is the simplest pattern of natural underwater polarization and the most easily imitated in the laboratory.

Verheijen (1958) and Waterman (1961) have emphasized that much of the experimental work on light reactions of aquatic animals has been conducted under conditions atypical of those in open water. In the previously described experiment, the plane of polarization was directed vertically from above the experimental cell. This experiment might simulate polarotaxis in shallow waters where overhead sky patterns are directly visible over about 90°. However, such conditions of polarization are limited to shallow waters and could not model the effect of lateral illumination from underwater polarization in deeper waters.

Methods

In order to approximate more natural conditions in the study of polarotaxis, the following setup was employed: Fifty copepods were placed in a rectangular battery jar, 156 mm. deep by 100 mm. long by 80 mm. wide. Sheets of Polaroid were placed on each side of the jar so that the plane of polarization was horizontal along all lines of sight at all times of day. This simulates the simplest natural pattern described above. The jar was illuminated from above by unpolarized light. Both the vertical and horizontal illumination was provided by tungsten filament lamps, but their intensities were set in two different patterns.

In surface waters the light intensity from above is much greater than the light intensity from the sides. To approximate this in the laboratory, the light sources were adjusted such that the intensity from above was 1100 lux inside the battery jar and the intensity from the sides was 100 lux inside the battery jar. As depth increases in natural waters, the ratio of the vertical to horizontal illumination decreases (Jerlov, 1951). To approximate the pattern of light intensity found in deeper water, the light intensities from above and from the sides were made equal inside the battery jar and set at either 1100 lux or 100 lux.

The intensity of light was controlled with a rheostat and measured with a luxmeter. Since changing intensity with a rheostat can produce changes in the wave-length of the emitted light, any behavioral changes induced by different intensity patterns might be due to different reactions of the organism to different wave-lengths of light. To establish that this was not the case, the intensities were also varied by changing the distance of the light source from the battery jar; this procedure does not change the wave-length. It was found that similar results were obtained in this set of experiments and in all others requiring differential intensities regardless of the manner in which intensity was varied.

The swimming copepods were observed visually and their paths were tallied in the following way: On a clear piece of plastic a circle was drawn and divided into 16 equal parts by 8 lines drawn as diameters through the center. One of these diameters was chosen to represent an up-down axis; the diameter perpendicular to it represented a left-right axis. The other diameters represented intermediate directions. When a copepod began swimming freely in the water, the center of this circle was visually aligned to the copepod's starting point so that one of the axes of the circle closely approximated the path of the swimming animal. The paths were then recorded over a period of a half hour and a frequency diagram plotted. The angle between the path of the copepod and the plane of polarization could be estimated only roughly with this method. Any given excursion of a copepod from rest into the open water had to be approximately aligned to one of the reference axes on the plastic sheet; the error involved could be as great as 11°, but was generally much less. The copepods were observed at approximately 6-hour intervals throughout the course of the 12-hour light period.

Results

Using horizontal polarization to simulate natural conditions, the effect of the angular distribution of light intensity on polarotaxis was studied (Fig. 2). With a contrasting ratio of vertical to horizontal intensity (1100 lux from above, 100 lux from the side), polarotaxis was always primarily 90° to the horizontal plane of polarization with no rhythmic responses present. However, with a uniform angular distribution of light intensity (equal intensity from both the sides and above) of either 1100 lux or 100 lux, a rhythm was present at both intensities. Compared with a control group where no polarization was present, these copepods swam more vertically (at right angles to the plane of polarization) at the beginning and end of the 12-hour light period and more horizontally (in the plane of polarization) at the middle of the light period. This indicates that a uniform angular distribution of light intensity over a range of absolute intensities induces the diurnal rhythm in the polarotactic response.

Swimming in unpolarized light is basically horizontal at all times of day, but orientation is not precise. This is true in uniform intensity patterns of either 1100 lux or 100 lux and in contrast intensity patterns as well. The addition of horizontally polarized light both increases the precision of horizontal swimming and induces a periodic vertical swimming component. The control group further demonstrates that there are no rhythms in orientation in unpolarized light. Polar-

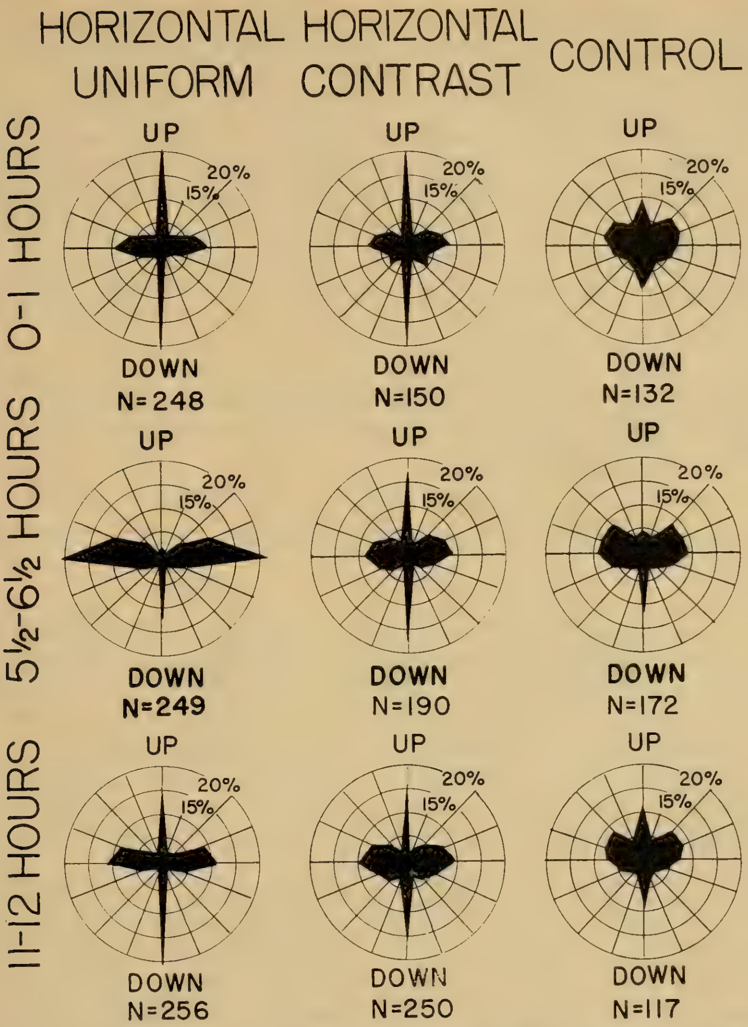

FIGURE 2. Orientation to horizontally polarized light at different times during a 12-hour light cycle. Radial axes represent direction of swimming with respect to gravity. Concentric circles represent the percentage of total swimming movements. N gives the total number of swimming movements. HORIZONTAL means horizontally polarized light was used; the plane of polarization is perpendicular to the UP-DOWN axis. UNIFORM means the light intensities from above and from the sides were equal. CONTRAST means the light intensity from above was greater than from the sides. CONTROL group had no polarization; angular distribution of intensity had no effect on this group.

ization must be present (along with a uniform angular distribution of intensity) to elicit a diurnal rhythm in the orientation behavior of these copepods. Furthermore, contrast and uniform angular distributions of light intensity elicit the same response (predominantly horizontal swimming) in unpolarized light, but each elicits a different response in polarized light.

In order to demonstrate conclusively that the observed rhythm in swimming behavior was not influenced by gravity responses, the lateral patterns of polarization were varied. The intensity in all cases was 1100 lux inside the battery jar from both above and from the sides. If the light from the sides was polarized in a vertical plane, instead of horizontally as above, the diurnal rhythm in polarotaxis still persisted, but now the copepods swam horizontally with respect to gravity (but still 90° to the plane of polarization) at the beginning and end of the light period and vertically with respect to gravity (but still 0° to the plane of polarization) midway through the light period.

If the plane of polarization was set at an angle of 45° to the horizontal on all sides of the jar, the coepods swam at an angle of 45° upwards and downwards with respect to gravity at all times of the day. The rhythm did not appear under these conditions since swimming at either 0° or at 90° to the plane of polarization both result in swimming 45° to the horizontal with respect to gravity. The frequency diagrams for these experiments are not figured in the paper since they can be adequately described verbally.

Polarotactic Behavior in Changing Intensities of Light

Introduction

All the experiments described so far indicate that polarized light merely orients swimming direction more precisely, either in a vertical, horizontal or 45° to the horizontal direction, but causes no net displacement of the population, *i.e.*, migration. Inspection of Figure 2 shows that given a population of *Cyclops vernalis* swimming primarily horizontally in unpolarized light, the addition of horizontally polarized light will alter the swimming behavior by inducing a vertically directed swimming component. However, this vertical component is equally divided into upward and downward fractions, causing no migration in the population.

Experiments on other planktonic organisms (Ewald, 1912; Clarke, 1930, 1932; Johnson, 1938; Johnson and Raymont, 1939) indicate that changing light intensities rather than constant illumination cause net displacements of the populations: increasing intensity causes a downward migration and decreasing intensity causes an upward migration when the light source is overhead. Similar changes have been observed in the deep scattering layer in nature (Hersey and Backus, 1962). This sound-scattering layer, composed of marine organisms, migrates upward at night and downward during the day, being greatly influenced by light intensity.

Methods

Since polarized light of constant intensity causes no migrations of the swimming *Cyclops vernalis,* the effects of intensity changes with and without horizontal polarization were investigated to see if net displacements could be elicited. Observations were made with either decreasing or increasing light intensities from above and/or from the sides to approximate the conditions at sunset and sunrise, respectively.

In cases of decreasing intensity, the initial intensity of light was 1100 lux and decreased by steps of 200 lux every ten minutes until a final intensity of 100 lux was reached. In cases of increasing intensity, the initial intensity was 100 lux and increased by steps of 200 lux every ten minutes until a final intensity of 1100 lux was reached. In addition, either the overhead and lateral lights changed intensity at the same rate (uniform angular distribution of intensity) or the lateral light remained constant at 100 lux while the overhead light changed in intensity (contrast angular distribution of intensity). The intensity of light was controlled manually with a rheostat and measured with a luxmeter as described previously.

Results

When polarization is not present, intensity changes caused no net displacements in the population; swimming remained primarily horizontal in all cases.

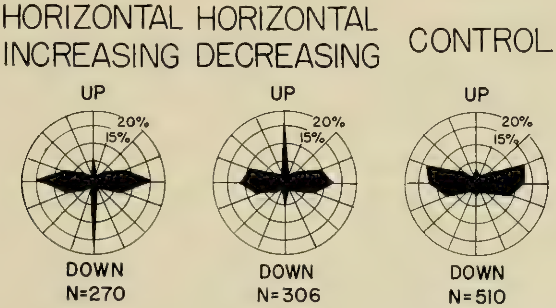

FIGURE 3. Orientation in changing light intensities. Method of diagramming is same as in Figure 2. HORIZONTAL means horizontally polarized light was used. INCREASING and DECREASING describe the change in light intensity. CONTROL group had no polarization; changing intensity of either type had no effect on this group. Time of day and pattern of intensity distribution had no effect on intensity responses.

Without polarization, then, orientation behavior in changing intensities was similar to that found with a constant intensity.

However, when the lateral light was polarized horizontally (Fig. 3), increasing intensity caused a net downward movement in vertically swimming individuals whereas decreasing intensity caused a net upward movement in vertically swimming individuals. Horizontally swimming individuals were not affected.

These results in horizontally polarized light held regardless of whether overhead and lateral lights changed intensity at the same or at different rates. The time of day also had no effect on the results; changing intensities always caused a vertical displacement of the population as long as polarized light was present. This indicates that the cue of changing intensity is stronger than the rhythm in polarotaxis that occurs when intensity is held constant: The 0° orientation to the plane of polarization that occurs at midday in a uniform intensity distribution of unchanging intensity is changed to a 90° orientation when changing intensities are employed. However, since intensities are normally constant at midday in nature, the rhythm probably persists under natural conditions.

Discussion

Ecological significance

From the experiments with *Cyclops vernalis* it is clear that experimental conditions including unpolarized light at constant intensity in all directions at all times of day are highly artificial. Extremely interesting patterns of behavior that would never occur under such highly artificial light regimes are elicited when certain aspects of natural conditions of illumination are simulated, even crudely. In order to understand the ecological significance of the various behavioral reactions of *Cyclops vernalis* to light, the various parameters of the experimental illumination must be given a natural interpretation.

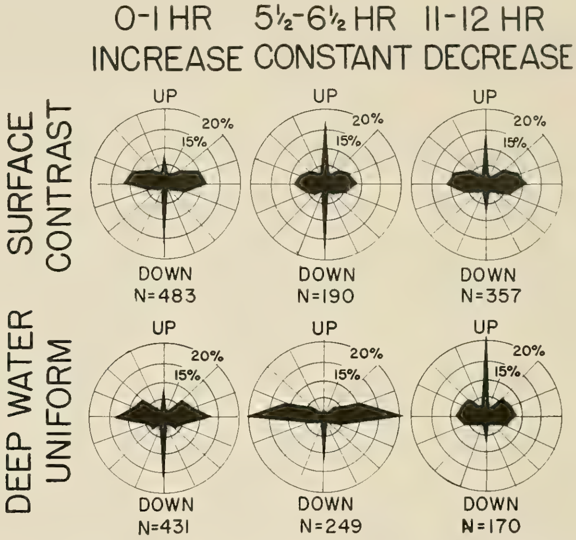

Figure 4. Summary of experiments using horizontally polarized light at different times of the 12-hour light cycle. Method of diagramming is same as in Figure 2. INCREASE, CONSTANT and DECREASE describe change in light intensity. CONTRAST describes intensity distribution found at the SURFACE of water. UNIFORM describes intensity distribution in DEEP WATER.

In this study, a horizontal polarization of light along all lines of sight was used to approximate the simplest of natural patterns of polarization in the aquatic habitat. It must be emphasized that this is a pattern that occurs only in low latitudes around noon. Increasing intensities of light were used to simulate conditions occurring during the early morning hours whereas decreasing intensities were used to approximate the conditions of illumination at dusk; constant intensities were used to represent conditions at midday. The effects of depth on light intensity patterns were crudely mimicked by changing the intensity ratio of the overhead and lateral lights and by the use of black or white sidewalls and diaphragm in the experiments using polarized light from above.

A summary of the experimental results is presented in Figure 4. Only those studies performed under conditions of illumination approximating those found in nature are used; those studies that elucidated the behavioral reactions but which have no natural counterpart (such as increasing intensities at the end of the light period) are omitted.

When all the experimental results are arranged in a natural sequence, the following trends appear: In the morning, swimming has a net downward component both at the surface and in deep water. A population behaving in this manner would tend to sink in the water. At midday, those copepods that sank into deep water in the morning would now move primarily horizontally in the water, maintaining their position in deep water. However, those copepods that remained at the surface during the morning would still tend to move vertically up and down at midday. Copepods moving vertically downward from the surface might eventually reach a more uniform angular distribution of light intensity, causing them to fan out horizontally.

In this way, the vertically swimming copepods found at the surface at midday would gradually be "trapped" by the deep-water angular distribution of light intensity, eventually causing this surface population to redistribute in deeper water. In the evening, swimming has a net upward component both at the surface and in deep water. This would cause the population to rise toward the surface in the evening. These trends in swimming behavior are consistent with the patterns of movement shown by many planktonic Crustacea in their vertical migrations. This implies that the parameters of illumination examined in this study may be very important for the orientation and release of migratory behavior in the aquatic habitat.

Physiological significance

If the foregoing interpretation of the experimental results with *Cyclops vernalis* is correct, then the physiological significance of the various parameters of illumination can be summarized as follows: The angular distribution of light intensity is a visual cue giving information as to the copepod's relative depth in the water. The plane of polarization is used as a frame of reference for orienting locomotor movements in the water where other orienting cues such as landmarks are sparse. By swimming at right angles to the plane of polarization, the copepod will move predominantly vertically at most times of day; by swimming in the plane of polarization, the copepod will move horizontally much of the time. Thus the polarization pattern of the aquatic habitat provides the copepod with vertical-horizontal reference coordinates with which to orient. Changing intensity of illumination provides migratory cues that lead to a net displacement of the population only when it is correctly oriented. Increasing intensity is a cue to swim downward, whereas decreasing intensity is a cue to move upward. The diurnal rhythm in polarotaxis that occurs only in deep-water uniform intensity distributions functions as a behavioral "trap" to keep the copepods in deep water during midday.

This investigation was supported by a National Aeronautics and Space Adminstration traineeship awarded through the Department of Biology at Yale University. Appreciation is expressed to Professor Talbot H. Waterman for the stimulation, guidance and comments that made this study possible.

SUMMARY

1. A series of laboratory experiments on the fresh-water copepod, *Cyclops vernalis,* revealed an endogenous rhythm in its orientation to polarized light. The copepod swam perpendicular to the plane of polarization at the beginning and end of a 12-hour diurnal light period, but swam parallel to the plane of polarization midway through this light period.

2. The rhythm was not present under all experimental conditions, being released only when the intensity of light from above was equal to that from the side. Under other experimental conditions, the copepods swam perpendicular to the plane of polarization at all times during the light period.

3. A pattern of horizontal polarization, crudely simulating the simplest natural underwater pattern, was found to provide *Cyclops vernalis* with vertical-horizontal reference coordinates for spatial orientation.

4. Changing intensities of polarized light induced net displacements of the population, thus effecting vertical migration in the laboratory.

5. The ratio of overhead to lateral light intensity provided a visual cue indicating water depth. The diurnal rhythm in polarotaxis operated only with deep-water intensity ratios of horizontal and vertical light and served to orient deep-water individuals in a horizontal plane at midday, thus inhibiting vertical swimming patterns (which occur at dawn and dusk) that might carry them to the surface at that time.

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POLAROTAXIS IN COPEPODS. II. THE ULTRASTRUCTURAL BASIS AND ECOLOGICAL SIGNIFICANCE OF POLARIZED LIGHT SENSITIVITY IN COPEPODS

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The ability to perceive linearly polarized light and to determine its plane of polarization has been demonstrated both behaviorally and electrophysiologically in a great number of species, mostly in arthropods but also in cephalopod mollusks (Waterman, 1966a). The mechanism of polarized light perception is localized at the retinal level and, in the cases adequately known, apparently depends on a two-channel system comprising a pair of dichroic analyzers oriented at right angles (Waterman, 1966b; Waterman and Horch, 1966; Eguchi and Waterman, 1966, 1967).

In a previous paper (Umminger, 1968), polarotaxis was behaviorally demonstrated in the copepod *Cyclops vernalis*. This was the first reported case of polarized light sensitivity in an organism possessing a naupliar eye exclusively. Earlier investigators (Stockhammer, 1959; Jander, 1966) had failed to find polarotaxis in copepods.

The purposes of this paper, then, are two-fold: First, an ultrastructural examination of the naupliar eye of *Cyclops vernalis* was undertaken to determine if there was a two-channel system of microvilli present as in polarotactic arthropods with compound eyes. Secondly, a great number of copepod species were examined for polarotactic behavior to see if the ability to perceive polarized light is universal or limited to only a few species of copepods. If polarotaxis is not universally present in copepods, this would explain the failure of Stockhammer and Jander to find it in the species they studied.

Fine Structure of the Naupliar Eye

Methods

To determine whether the polarotactic behavior of *Cyclops vernalis* Fischer could be explained by some structural component of the naupliar eye, the fine structure of the eye was examined. The entire copepod was first fixed for two hours with 5% glutaraldehyde buffered in 0.1 *M* Sorenson's phosphate solution at pH 7.4 (Sabatini, Bensch and Barrnett, 1963). After washing with buffer, the copepods were cut in half and the metasomes post-fixed for another two hours in 2.5% osmium tetroxide, also buffered at pH 7.4 with Sorenson's solution. Fixation was followed by acetone dehydration, transfer to propylene oxide and embedding in epoxy resin (Luft, 1961). Sections were cut with a Porter-Blum MT-2 microtome and stained with uranyl acetate and lead citrate (Reynolds, 1963). Observations were then made in a Philips 200 electron microscope.

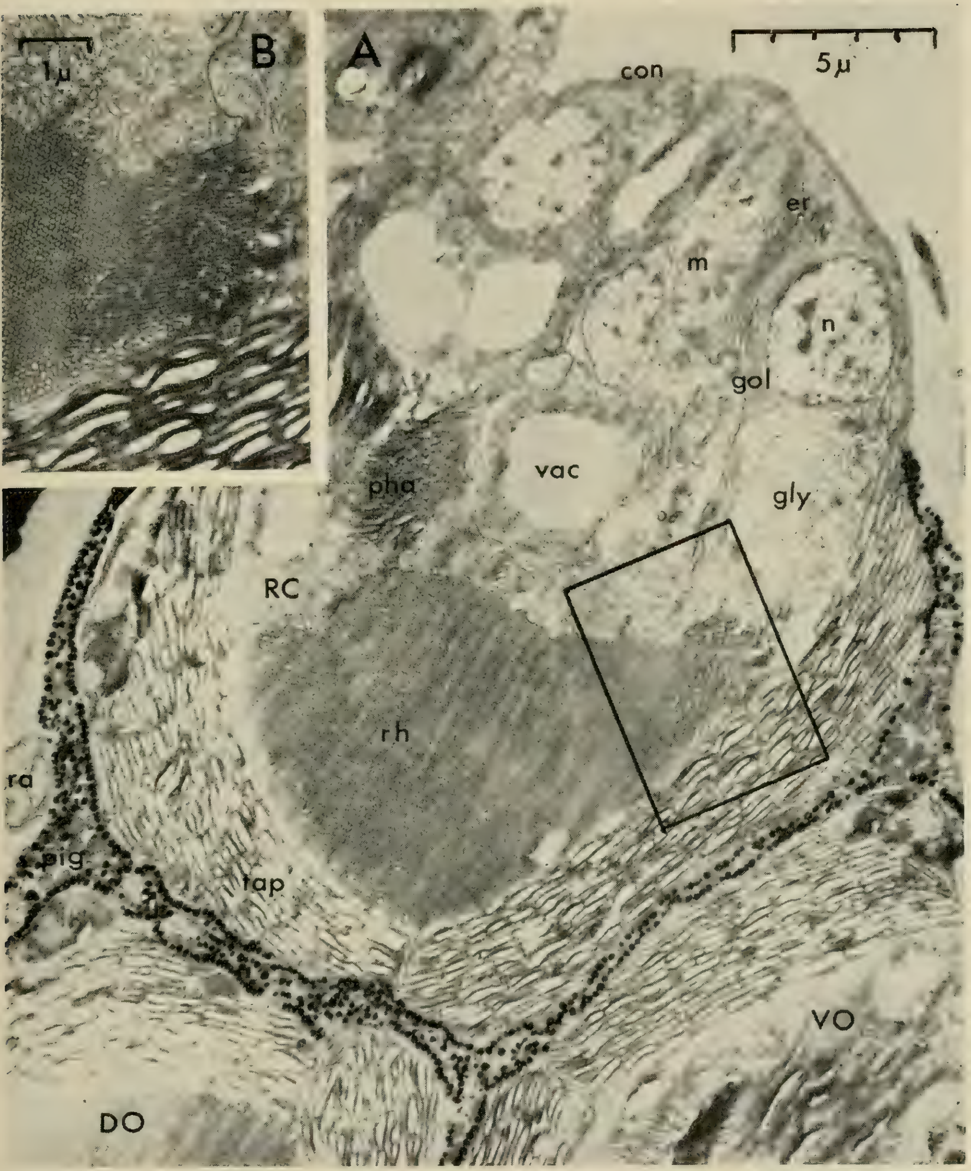

FIGURE 1. Longitudinal section through a dorsal ocellus of *Cyclops vernalis*. A. Portions of the other dorsal ocellus, DO, and the single ventral ocellus, VO, can be seen at the bottom. The two dorsal ocelli and the ventral ocellus make up the tripartite naupliar eye. con = conjunctiva, er = endoplasmic reticulum, gly = glycogen granules, gol = Golgi complex, m = mitochondrion, n = nucleus, pha = phaosome, pig = pigment cell, ra = retinular cell axons, RC = retinular cell, rh = rhabdom, tap = tapetum, vac = vacuole. Box represents area enlarged in B. B. Higher-power detail of the rhabdom.

Results

The "eye" of *Cyclops vernalis,* as is typical of most copepods, is composed of three ocelli on the anterodorsal region of the protocerebrum. The two dorsal ocelli each measure about 25 μ in diameter and point in a lateral direction. The single ventral ocellus points ventrally towards the rostrum.

The dorsal ocellus (Fig. 1) is composed primarily of several retinular cells whose inner ends have projections of microvilli forming the light-sensitive rhabdom. Of particular interest is the arrangement of these microvilli: those of one retinular cell meet those of another retinular cell at right angles (Fig. 1B). The retinular cells contain phaosomes, much endoplasmic reticulum and many glycogen particles. A reflecting tapetum forms a hemispherical cup around the bases of the light-sensitive retinular cells whereas a thin conjunctiva covers their distal surfaces. Surrounding the tapetum, which is tightly packed with crystals, is a screening pigment cell containing granules of melanin.

Light enters the ocellus through the conjunctiva and passes through the distal parts of the retinular cells. It then strikes the rhabdom which has its microvilli perpendicular to the incoming light.

POLAROTACTIC BEHAVIOR IN COPEPODS

Material and Methods

In an attempt to discover whether polarotaxis is widespread in copepods, a number of species from several orders of the Subclass Copepoda were examined. Several of the species studied had simple naupliar eyes as does *Cyclops vernalis,* whereas others had elaborately modified naupliar eyes, often with cuticular lenses and movable parts (Vaissière, 1961).

In experiments performed at the Duke University Marine Laboratory, the copepods were freshly captured each day and examined at times of natural sunrise, midday and sunset; none were cultured nor kept on a 12-hour light, 12-hour dark cycle as was *Cyclops vernalis* (Umminger, 1968). At Yale University, specimens of *Tisbe furcata* (Baird) were cultured and kept on a 12-hour light, 12-hour dark cycle of illumination. In addition, the fresh-water *Diaptomus shoshone* Forbes was studied after it had been flown to New Haven from Colorado.

All species were tested with linearly polarized light in experiments identical to those performed on *Cyclops vernalis* in a previous paper (Umminger, 1968).

Results

Polarotactic behavior like that of *Cyclops vernalis* could not be elicited in all the species studied. Several species oriented to polarized light whereas other species showed no polarotactic responses whatsoever. Using linearly polarized light from above, *Caligus rapax* Milne Edwards, *Centropages hamatus* (Lilljeborg) and *Diaptomus shoshone* Forbes all swam at right angles to the plane of polarization. *Pontella meadii* Wheeler and *Tisbe furcata* (Baird) both oriented 0°, ±45° and 90° (4 peaks) to the plane of polarization. *Labidocera aestiva* Wheeler oriented 0° to the plane of polarization. In all these species, however, the angle of orientation was the same at all times of day and under all conditions of illumination. There were no rhythms in polarotaxis.

The species showing no polarotactic responses were *Pseudodiaptomus coronatus* Williams, *Acartia tonsa* Dana, *Eucalanus monachus* Giesbrecht, *Corycaeus speciosus* Dana, *Oncaea venusta* Philippi, *Oithona similis* Claus, *Oithona nana* Giesbrecht, *Oithona spinirostris* Claus and *Euterpina acutifrons* (Dana).

DISCUSSION

Polarized light perception and eye structure

In many arthropods, particularly Crustacea, and cephalopod mollusks, where polarized light perception has been best demonstrated, there is always a regular arrangement of two sets of retinal microvilli which comprise the rhabdom, one set being perpendicular to the other (Eguchi and Waterman, 1966; Waterman, 1966c). If the dichroic molecules of visual pigment are oriented so that their major axis of absorbance is parallel to the long axis of the microvilli, then the orthogonal arrangement of microvilli functions as a two-channel retinal analyzer of polarized light (Waterman, 1966b; Waterman and Horch, 1966; Eguchi and and Waterman, 1967). Thus the set of retinular cells with the long axes of their microvilli parallel to the plane of polarization will be maximally stimulated by the incoming polarized light, whereas the remaining retinular cells with microvilli having their long axes perpendicular to the plane of polarization will be minimally stimulated. If both sets of microvilli are at a 45° angle to the plane of polarization, then both will be stimulated to the same degree. If the organism compares the stimulation in one set of microvilli to the stimulation in the other set, it has instantaneous information as to its orientation with respect to the plane of polarization.

However, if an organism has only one set of parallel microvilli, it might also orient to polarized light, but it could not get an instantaneous bearing. In this case, the organism would have to compare the stimulation received by this one set of parallel microvilli at one point in time with the stimulation received at another point in time. Thus, if the organism were oriented so that the long axes of the microvilli were parallel to the plane of polarization, the retinal stimulation would be maximal. Then, if the organism tilted or turned a bit, the microvilli would no longer be parallel to the plane of polarization, thus reducing retinal stimulation. The comparison of successive stimulations in time would allow the organism to orient so that retinal stimulation was maximal; in this way the microvilli would always be parallel to the plane of polarization. However, a very real disadvantage to this system is that changing light intensities might not be discriminated from the *e*-vector responses. A decrease in light intensity might confuse the organism if it interpreted such a change as a deviation from its position of orientation to the plane of polarization. One would expect, then, that such a one-channel system is not ideally suited for polarotactic behavior.

The fine structure of the eye of *Cyclops vernalis* clearly shows regions of mutually perpendicular microvilli, suggesting that this geometric arrangement is responsible for the copepod's perception of polarized light. Furthermore, both sets of microvilli are perpendicular to the incoming light as is typically the case of compound or camera eyes sensitive to polarized light. When the copepod is swimming in either of its usual positions (horizontally or vertically), the laterally pointing dorsal ocelli (Fig. 1) will be oriented so that one set of microvilli is parallel to the naturally horizontal plane of polarization whereas the other set is perpendicular to this plane. Therefore, the vertical and horizontal directions of the microvilli correspond to the vertical and horizontal axes of the copepod's normal spatial orientation, a situation closely similar to that in decapod crustacean compound eyes (Waterman and Horch, 1966; Eguchi and Waterman, 1967) and in cephalopod camera eyes (Tasaki and Karita, 1966).

Unfortunately, no electron micrographs are available for the other species of copepods studied, but light microscopy by Vaissière (1961) suggests that *Centropages typicus* has parallel rows of microvilli in its naupliar eye whereas *Corycaeus clausi* has no regular arrangement of microvilli, the entire rhabdom being curved so that the microvilli are not parallel. In the experimental studies presented here, *Centropages hamatus* is polarotactic whereas *Corycaeus speciosus* is not. This additional information suggests that polarotactic behavior is correlated with a

TABLE I

Relation of polarotaxis to copepod ecology

Species	Polarotactic	Medium	Habitat	Vertical migration	Food habits	Eye structure
Calanoida						
Labidocera aestiva	Yes	SW′	Pelagic*	Yes††	Omnivore[@@]	Complex
Pontella meadii	Yes	SW	Pelagic*	—	Omnivore[+]	Complex
Centropages hamatus	Yes	SW	Pelagic*	Yes[φ]	Omnivore[@@]	Complex
Diaptomus shoshone	Yes	FW″	Pelagic**	Yes[φφ]	Predator**	Simple
Pseudodiaptomus coronatus	No	SW	Pelagic*	Yes[#]	Probably herbivore	Simple
Acartia tonsa	No	SW	Pelagic*	Yes[##]	Omnivore[@@]	Simple
Eucalanus monachus	No	SW	Pelagic*	—	Probably herbivore	Simple
Cyclopoida						
Cyclops vernalis	Yes	FW	Littoral†	—	Predator[++]	Simple
Corycaeus speciosus	No	SW	Pelagic*	Yes[¢]	Predator[+]	Complex
Oncaea venusta	No	SW	Pelagic*	—	Probably herbivore	Simple
Oithona similis	No	SW	Littoral*	Yes[¢¢]	Predator[$]	Simple
Oithona nana	No	SW	Pelagic*	Yes[φ]	Herbivore[$$]	Simple
Oithona spinirostris	No	SW	Pelagic*	Yes[@]	Predator*	Simple
Harpacticoida						
Tisbe furcata	Yes	SW	Littoral*	—	Herbivore[=]	Simple
Euterpina acutifrons	No	SW	Pelagic*	—	Scavenger[::]	Simple
Caligoida						
Caligus rapax	Yes	SW	Pelagic*	—	Exoparasite*	Complex

′ Sea water; ″ fresh water.

*Wilson, 1932; **Brooks, J. L., personal communication; †Pennak, 1966; ††Parker, 1902; [φ]Hansen, 1951; [φφ]Pennak, 1944; [#]Grice, 1957; [##]Schallek, 1942; [¢]Roehr and Moore, 1965; [¢¢]Bogorov, 1946; [@]Moore and O'Berry, 1957; [@@]Anraku and Omori, 1963; [+]Personal observation; [++]Dziuban, 1964; [$]Marshall and Orr, 1966; [$$]Murphy, 1923; [=]Battaglia and Bryan, 1964; [::]Lebour, 1923.

geometrically regular arrangement of microvilli within the eye, a condition essential to the dichroic rhabdom analyzer mechanism.

Of the copepods studied, five had very elaborate naupliar eyes with cuticular lenses and often with movable parts (Table I). Of these five species with such complex eyes, four gave evidence of perceiving polarized light. Of the eleven species with simple naupliar eyes, only three responded specifically to polarized light. Since polarized light can be perceived by both simple and complex naupliar eyes and since its perception clearly does not depend on lenses or other gross structural modifications, then this again points to the ultrastructure of the eye as the site of polarized light perception. Actually, the tendency for polarized light perception to be more prevalent in forms with complex eyes may be explained by the fact that all these species are predators (Table I).

Polarized light perception by copepods

Although polarized light sensitivity has been reported for cephalopod mollusks with camera eyes (Moody and Parriss, 1960, 1961; Jander, Daumer and Waterman, 1963; Tasaki and Karita, 1966), for arthropods with compound eyes (Baylor and Smith, 1953; Jander and Waterman, 1960; Waterman, 1966a) and for insect larvae (Wellington, 1953, 1955; Waterman, 1966a), mites (Baylor and Smith, 1953) and spiders (Papi, 1955; Waterman, 1966a) with simple ocelli, it has only once been reported for an organism with a naupliar eye exclusively (Umminger, 1968). However, since Stockhammer (1959) was unable to find polarotaxis in copepods of the *Cyclops* group and since Jander (1966) could not demonstrate polarized light sensitivity in the copepods *Macrocyclops albidus, Cyclops strenuus* and *Labidocera* spp., it appeared that at least some copepods did not exhibit polarotaxis. The present paper confirms definitely that the naupliar eye can perceive polarized light, but that this ability is not to be found universally in copepods.

The failure of Stockhammer and Jander to find polarotaxis in copepods could be due either to organismal or to experimental factors. In the former case, the species investigated actually may not have been able to perceive polarized light. Since the structure of the naupliar eye varies so greatly with the Crustacea as a whole (Elofsson, 1963) and even within the Copepoda (Vaissière, 1961), it is entirely possible that the species examined by Stockhammer and Jander did not possess eyes with the regular two-channel arrangement of microvilli necessary for polarized light perception. This appears to be the reason why *Corycaeus speciosus* showed no polarotaxis in the present paper.

For example, Fahrenbach (1964) has made an ultrastructural study of the eye of *Macrocyclops albidus.* The eye contains great masses of microvilli running parallel, but not perpendicular, to one another in a tightly packed array. As previously discussed, such a one-channel system is not ideally suited for polarized light detection and, indeed, Jander (1966) observed no polarotaxis in this species.

Moreover, even if a species did possess the appropriate eye structure and did perceive polarized light, it is entirely possible that the copepods did not behaviorally react to it because they do not use polarized light as a sensory cue in their orientation behavior; *i.e.,* they perceive, but ignore, polarized light.

In addition to such organismal factors, several experimental factors might also explain the failure of Stockhammer and Jander to observe polarotaxis in copepods. Thus the time of observation may have been wrong. That is, there may have been a rhythm in polarotaxis such that observations were made only during the unoriented phase of behavior. This explanation seems likely for those copepods of the *Cyclops* group. If other members of the genus *Cyclops* show a rhythm as does *Cyclops vernalis* (Umminger, 1968), then orientation peaks would appear at dusk, midday and dawn, the times when most investigators are least likely to be observing copepods. At other times, the copepods would be in a transitional state and appear unoriented.

Another experimental factor which may explain the failure to obtain polarotactic responses is as follows: The copepods may have sensed the polarized light, but the correct combination of illumination parameters (absolute intensity, wave-length, angular distribution of intensity, changing intensity, etc.) was not employed to release the corresponding polarotactic behavior.

Ecological significance of polarotaxis

In a previous paper, a polarotactic rhythm in *Cyclops vernalis* occurred in an ecologically meaningful way, implicating it as a behavioral mechanism in vertical migration (Umminger, 1968). In the present paper, however, all the copepods with polarotactic behavior showed no rhythm in their responses. This may be due either to the absolute lack of such rhythms or to the failure of the experiments to provide the correct combination of illumination (or other) parameters needed to release the rhythm. In the experiments with *Cyclops vernalis,* for example, the rhythm was released only in the presence of a uniform angular distribution of light intensity. If such an esoteric cue is needed as a prerequisite for the rhythm in this case, then equally unsuspected conditions may be necessary to release the rhythms in the other copepods with polarotactic behavior.

Moreover, as discussed above, the lack of demonstrated polarotaxis in other species of copepods examined in this paper does not necessarily mean that the copepods in question cannot perceive linearly polarized light, but may indicate the failure of the experimental conditions to elicit a response to it. On present evidence, however, one must assume that orientation rhythms are not present in some copepods with polarotaxis and that other copepods do not have polarotactic behavior.

If one accepts the observed differences in polarotaxis as real, then some clue as to their ecological significance might come from comparing the ecologies of polarotactic and non-polarotactic species (Table I).

Obviously polarotaxis has no relation to habitat since it is found in both salt-water and fresh-water forms and is common in both pelagic and littoral copepods. Furthermore, natural vertical migrations have been reported in forms without polarotaxis as well as in forms with polarotaxis. Therefore, although polarotaxis may be useful for orientation in vertical migration as with *Cyclops vernalis,* polarized light sensitivity may not be necessary for such migrators.

One rather striking similarity among polarotactic copepods is their tendency to be either predatory or omnivorous. Of the seven copepods showing polarized light sensitivity, six are either predators or omnivores. This suggests that the ability to perceive polarized light might be of greater advantage to a copepod with predatory habits than to one that is strictly herbivorous. This may be related to the findings of Lythgoe and Hemmings (1967) who reported that polarized light sensitivity in clear waters enables distant objects to be more clearly seen and causes contrasts in intensity to be much sharper. Such an improvement in visual ability would seem to be more advantageous to a predator than to an herbivore. One might expect, then, the findings that polarized light sensitivity is most common in predators.

The improved visual ability imparted to predators by polarized light sensitivity does not preclude, however, the additional use of this sensitivity for spatial orientation and migration. The very fact that the predators actively orient to the plane of polarization demonstrates that they do more than passively perceive it; they use it to modify their spatial orientation as well.

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SUMMARY

1. The ability of *Cyclops vernalis* to perceive polarized light has an ultrastructural basis in the presence of mutually perpendicular microvilli in its naupliar eye.

2. Laboratory investigations with several species of copepods were conducted to determine the extent of polarized light sensitivity in this group. Polarotaxis was not found to be universally present in copepods, but apparently depended on the presence of mutually perpendicular microvilli in the rhabdom of the naupliar eye.

3. Polarotaxis showed no correlation with the presence of a complex, rather than simple, naupliar eye.

4. The ability to perceive polarized light was more prominent in predatory than in herbivorous species, suggesting that its function might be to enable the copepods to discern distant objects more clearly, in addition to its role in orientation during vertical migration.

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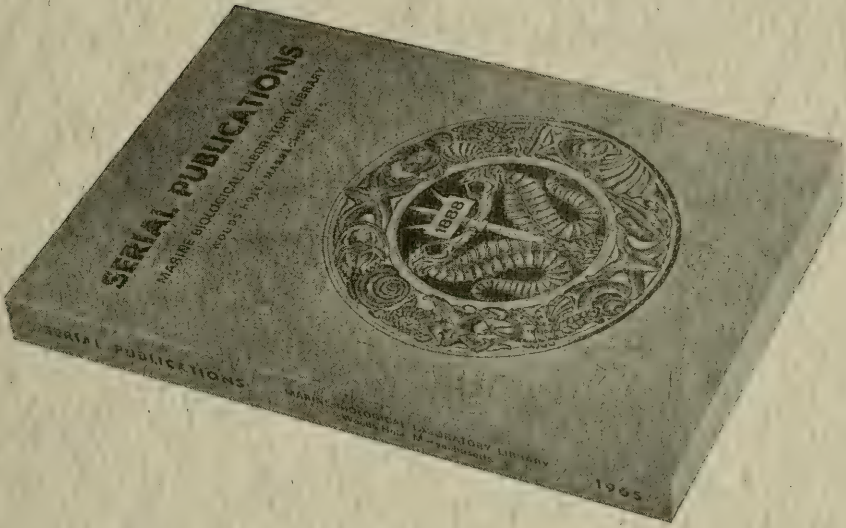

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CONTENTS

Page
Annual Report of the Marine Biological Laboratory 1

ARME, C., AND C. P. READ
Studies on membrane transport. II. The absorption of acetate and butyrate by Hymenolepis diminuta (Cestoda) 80

BENNETT, MIRIAM F., AND JUDITH A. HARBOTTLE
The effects of hydrocortisone on the blood of tadpoles and frogs, Rana catesbeiana 92

CAMPBELL, RICHARD D.
Colony growth and pattern in the two-tentacled hydroid, Proboscidactyla flavicirrata 96

CHENEY, DANIEL P.
Red cell morphometrics and volume in the cichlid teleost Tilapia mossambica, using a chromium-51 labeling method 105

GORE, ROBERT H.
The larval development of the commensal crab Polyonyx gibbesi Haig, 1956 (Crustacea: Decapoda) 111

HASCHEMEYER, AUDREY E. V.
Compensation of liver protein synthesis in temperature-acclimated toadfish, Opsanus tau 130

JEFFREY, S. W.
Pigment composition of Siphonales algae in the brain coral Favia . . . 141

JEFFREY, S. W., AND F. T. HAXO
Photosynthetic pigments of symbiotic dinoflagellates (zooxanthellae) from corals and clams 149

KRIEBEL, MAHLON E.
Pacemaker properties of tunicate heart cells 166

KRIEBEL, MAHLON E.
Cholinoceptive and adrenoceptive properties of the tunicate heart pacemaker 174

LOFTFIELD, R. B., E. A. EIGNER AND J. NOBEL
Inter-phylogenetic specificity in the bonding of amino acids to tRNA 181

MARSH, JULIAN B.
Isolation and composition of a low density lipoprotein from the eggs of Arbacia punctulata 193

NIU, M. C., AND A. LEIKOLA
The developmental potentiality of the liver-RNA-treated posterior primitive streak in the chick embryo 200

POTSWALD, HERBERT E.
The biology of fertilization and brood protection in Spirorbis (Laeospira) morchi 208

RIGBY, B. J.
Temperature relationships of poikilotherms and the melting temperature of molecular collagen 223

SHOOP, C. ROBERT
Migratory orientation of Ambystoma maculatum: Movements near breeding ponds and displacements of migrating individuals 230

UMMINGER, BRUCE L.
Polarotaxis in copepods. I. An endogenous rhythm in polarotaxis in Cyclops vernalis and its relation to vertical migration 239

UMMINGER, BRUCE L.
Polarotaxis in copepods. II. The ultrastructural basis and ecological significance of polarized light sensitivity in copepods 252

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BIOLOGICAL BULLETIN

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THE LARVAE OF A DISCINID (INARTICULATA, BRACHIOPODA)

SHOU-HWA CHUANG

Department of Zoology, University of Singapore, Singapore 10

The first account of a discinid larva was given by Mueller (1860). This larva from the sea off Santa Catarina, Brazil, had a pair of thin, approximately circular valves with no hinge, a lophophore with a median tentacle and 4 pairs of cirri, a pair of eyespots, a pair of statocysts, a pedicle, 5 pairs of stout mantle setae and a large number of fine mantle setae. Further specimens (Mueller, 1861), also with 4 pairs of cirri, remained free-swimming for about 5 or 6 days before they became attached to the bottom and sides of glass vessels in the laboratory.

Simroth (1897) reported two inarticulate larvae, one with 3 pairs of cirri and two bunches of extremely fine setae and the other with 4 pairs of cirri and 3 widely separated stout mantle setae. The former, he believed (p. 4), would not develop any more cirri during its pelagic larval life. This belief, the morphological differences between the two larvae and the fact that one was obtained near the surface and the other from a depth of 1000 to 2000 meters, led him to the conclusion that one was adapted to planktonic life at the surface, and the other to life at great depth (p. 6). He further concluded (p. 5) that the larvae belonged to two different species of a single discinid genus.

Blochmann (1898) obtained 10 larvae from the Strait of Rhio in the Pacific identical with Mueller's larvae both in structure and in the stage of development. He considered them as larvae of *Pelagodiscus atlanticus* (King) since the adults of this worldwide species were reported from both the Atlantic and the Pacific. However, in his alum-carmine-stained specimens, he only found nephridia on either side of the stomach where Mueller (1860) had previously seen only statocysts.

Yatsu (1902) described a discinid larva off Japan with a pair of statocysts, 5 pairs of stout setae and a large number of fine mantle setae. He mentioned the possibility (p. 107) that the free-swimming discinid larvae at the stage of 4 pairs of cirri made their way towards the coast shortly before settlement.

Eichler (1911) obtained two large larvae from a depth of 3000 meters at 66 degrees S. Lat. and 90 degrees E. Long. These also had 4 pairs of very long cirri, a pair of statocysts, 4 pairs of stout setae and a great number of fine setae. He also

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attributed these to *Pelagodiscus atlanticus,* adding (p. 384) that they had given up pelagic life and were changing over to a sessile mode of life.

Ashworth (1915) found 6 larvae of *Pelagodiscus* from a depth of 40 fathoms in the Indian Ocean. These also had 4 pairs of cirri and led him to believe (p. 66) that the younger stages might live on the sea bottom and hence escaped capture.

In the present study a complete series of larvae was obtained. The smallest was even smaller than the smallest ever reported (Simroth, 1897) and the largest exceeded in size all the largest larvae ever reported, except for the two of Eichler (1911). Moreover, the larvae of medium size in the present series showed the transitional stages between the two larvae described by Simroth (1897), thus serving to provide evidence for the probable identity in species of the two larvae of Simroth. The nature of the organs lying on either side of the stomach was also studied, since these were differently interpreted by Mueller (1860) and Blochmann (1898). The morphological changes during the larval stage were described. The larvae were compared with those previously found and their similarities and differences discussed.

Materials and Methods

Thirty-three larvae of a discinid were obtained by surface plankton hauls carried out in the daytime in the shallow water of the Johore Strait off the north coast of Singapore Island. Two of them were caught on August 28, 1952 (Table I, nos. 15 and 17), one each on September 21, 1952 (not in Table), September 28, 1952 (Table I, no. 2) and June 12, 1953 (Table I, no. 1). The others, which were obtained on November 16, 1952, formed a continuous series of transitional developmental stages and are presumed to belong to the same species. Before the adults are discovered in the vicinity of Singapore, these larvae are provisionally identified as those of a discinid on the grounds of the resemblance of some of the stages in the present series to similar larvae reported by Mueller (1860), Simroth (1897) and others.

The shell and setae of each larva were measured with a calibrated ocular micrometer under a monocular microscope. Shell dimensions were taken with the larva lying on its dorsal or ventral valve. The shell length was the shortest distance between the anterior and posterior edges of the shell along the sagittal plane, when both edges were in the same plane of focus. Similarly, the width of the shell was the distance between the right edge and the left edge at the widest part of the shell when both edges were in focus.

The number and type of setae and the number of paired cirri in the larvae were recorded. The absence or presence of statocysts was noted.

All observations were carried out on live specimens in the Department of Zoology, University of Singapore.

Results

A moderately young discinid larva consisted of a dorsoventrally flattened body enclosed between two shell valves. These were slightly convex externally, not hinged but merely held together by muscles and other soft tissues. The visceral cavity occupied a small central part in the posterior half of the space between the

TABLE I

Shell dimension and the type, number and length of setae in discinid larvae with 4 pairs of cirri from the Johore Strait

Speci-men no.	Shell valve				Length in μ of embryonic seta										Type and number of setae		
	Dorsal		Ventral		No. 1		No. 2		No. 3		No. 4		No. 5		Embryonic	Early larval	Late larval
	Length μ	Width μ	Length μ	Width μ	Left	Right	Left	Right	Left	Right	Left	Right	L.	R.	Left Right	Left Right	Left Right
1	462	500	349	455											0	1, 1	24, 24
2	401	479	337	434											0	1, 1	0
3	382	437	326	407											0	2, 2	0
4			347	429													
5			346	424													
6	311	364	291	364											0	4, 5	0
7	311	364	288	356											0	2, 2	0
8	308	358															
9	282	330	265	323		1213		1289							0, 2	3, 3	0
10	271	326	259	318	1198		1213,	1243							2, 1	3, 3	0
11	253	302	245	297	1107		1228								2, 0	2, 2	0
12	242	284	239	276	1152,	1198	1167,	1213				917			2, 3	0	0
13	241	288	243	288	121,	1137	1152,	1122							0	3, 2	0
14	233	259	219	251											1, 2	2, 2	0
15	217	242	212	238		773		1228		819					3, 3	(1), (1)	0
16	216	251	208	245	1092,	1092	1084,	1084	819,	834	857,	834			4, 4	3, 3	0
17	216	236	197	232													
18	213	242	207	236	1152,	1152		1289			857,	857			2, 3	3, 3	0
19	207	239	199	228	1152,	1122	1228,	1198			925,	917			3, 3	0	0
20	184	212	179	204					796,	781	819,	803			2, 2	3, 3	0
21	179	199	173	193		1167		1213							0, 2	0	0
22	179	196			1092,	1107	1182,	1137	788		758				4, 2	0	0
23	168	183	156	173	1175,	1152	1175,	1167		872					2, 3	0	0
24	161	183	160	184	1198,	1213	1243,	1228	773,	773		766	99,	83	4, 5	1, 2	0
25			157	169		1031				803	773,	773			1, 3	0	0
26	158	173	147	161	1213,	1182	1243,	1243		788		773			2, 4	0	0
27	143	151	130	138	1122,	1137	1228,	1198	879,	879	940,				4, 3	0	0
28	140	145	130	136					788		803,	834			2, 1	0, 1	0
29	124	125	104	111		1137	1228,	1228	819,	849	849,	819			3, 4	0	0
30	115	124	112	118		1084	1122,	1152			948,	955			2, 3	0	0
31	108	115	98	99			1243,	1228	894,	894	925				3, 2	0	0
32					1078,	1078	1038		728		697				4, 1	0	0

shell valves and was almost filled with the stomach and the intestine (Fig. 1). A cone of tissue projected laterally from the lateral body wall at the level of the stomach. This setal cone contained the bases of the long straight, brittle setae, which will be referred to as the embryonic setae in the present study. This cone also housed the muscles to move these setae.

The rest of the space between the shell valves not occupied by the visceral cavity constituted the mantle cavity. In the front part of this was a contractile columnar projection of the anterior body wall. This column contained the oesophagus, pharynx and muscles and continued anteriorly into the lophophore. Muscular movements of the column and lophophore brought about extension and expansion or retraction and contraction of both column and lophophore.

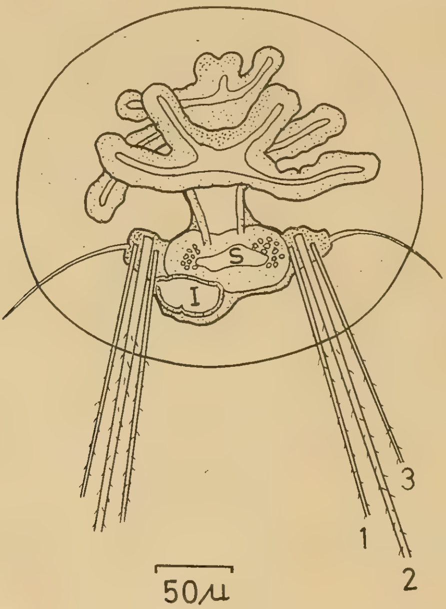

FIGURE 1. Ventral view of a young discinid larva (no. 15, Table) with embryonic setae (1–3) and a curved seta issuing from each setal cone. The stomach (S) and intestine (I) almost fill up the visceral cavity.

The larval lophophore consisted of a central disk roughly horizontal in position with the more or less centrally placed mouth on the ventral side. At the periphery of the disk 9 lophophoral processes radiated out. One of these, the tentacle, pointed anteriorly in the sagittal plane, and the others, the cirri, were more lateral in position. All the larvae in the present series had 4 pairs of cirri. Each cirrus on one side of the mouth usually corresponded in position and size with the one directly opposite on the other side, thus forming a pair.

A thin layer of mantle tissue lined the inner surface of the mantle cavity. In widely separated regions along the lateral border of the ventral mantle typically 4 stout curved setae appeared on each side of the medium-sized larvae. From each lateroposterior border of the dorsal mantle appeared another. These 5 curved setae will be referred to as the early larval setae.

In the oldest larvae yet another kind of setae appeared in the lateral border of the mantle. They were shorter and more flexible than the other two types and will be referred to as the late larval setae.

The lengths and widths of both the dorsal and the ventral shell valves were tabulated in Table I, in which the larvae were arranged in descending order of magnitude of the length of the dorsal shell valve wherever possible. Data on the different kinds of setae on the left and the right side of the body were also included.

Both shell valves were transversely oval in shape in the youngest larvae, although occasionally one valve or the other was circular, *e.g.,* the dorsal valve of no. 29 and the ventral valve of no. 31 in Table I. However, while the dorsal shell valve maintained approximately the same oval shape during the entire larval stage, the ventral one changed shape. The convex posterior edge of this in the youngest larvae gradually became less and less curved until it became almost a straight line when the larva attained about 219 μ in ventral valve length (no. 14). The posterior edge remained straight even at 243 μ ventral shell-valve length (no. 13), but with further growth it gradually developed a slight but noticeable bay near the median line, when the ventral-valve length reached 265 μ or so (no. 9). This bay became more prominent with further larval growth to 326 μ (no. 3) and deeper still in larger larvae.

This change in shape of the ventral shell valve was presumably caused by a slowing down of deposition of shell material at the posterior end. The deceleration was greatest near the sagittal plane, becoming less laterally.

With the exception of larvae nos. 13 and 24, in both of which the two shell valves were equal in both length and width, and of no. 6 in which the two shell valves were equal in width, all the larvae had unequal shell valves. In each of them the dorsal shell valve was slightly longer and wider than the opposing ventral one. This difference was slight throughout this series with the exception of the largest larvae (nos. 1 to 3), in which the difference was considerable.

The external surface of both shell valves was smooth. There was no observable growth line with the exception of larva no. 2. In this there was an oval area, 168 μ long and 183 μ wide, which appeared as a prominent growth line on the dorsal shell valve.

In the smallest larva (no. 31 in Table I) the alimentary canal consisted of the mouth, pharynx or oesophagus, stomach, intestine and the anus. In an older larva such as no. 15 the intestine was constricted near the distal end to form another chamber. The highly extensible oesophagus was long and slender when the lophophore was everted during swimming. When this was retracted the inner end of the oesophagus projected into the stomach in the form of a valve-like fold, almost completely closing the opening of the oesophagus into the stomach. The stomach was a large sac with its lateral diameter greater than its anteroposterior one. The high epithelium laterally indicated distinct regions of phagocytosis. The intestine was a thin-walled tube with a distinct constriction separating it from the stomach. It was directed to the right lateroposteriorly in young larvae but either laterally or anterolaterally in older specimens. The anus, not easily distinguishable except during defecation or after, was a slit about 2 to 2.5 μ in diameter.

Defecation was observed in larva no. 19. It was preceded by a flattening of the stomach in the anteroposterior direction. The stomach and the intestine then shrank visibly and progressively as particles from the hind part of the intestine escaped through the anus. Meanwhile the constriction between the stomach and the intestine also diminished in diameter. Presumably during defecation muscular contraction of the wall of both the stomach and the intestine was responsible for the expulsion of the faecal material.

Statocysts, absent in young larvae, occurred in the oldest larvae nos. 1 to 3. Each of these had a statocyst situated dorsally in the visceral cavity at each posterolateral corner of the stomach. Each statocyst had a central cavity, in which danced 15 to 25 statoliths. The cilia on the inner epithelium of the statocysts were also observed to stir vigorously.

The pedicle occurred in larva no. 6 and in the larger larvae as an oval vesicle posterior to the stomach. It was absent in younger larvae.

The embryonic setae were the first to appear among the three different kinds of setae found during the larval stage in a discinid. They were observed in young larvae of dorsal shell length of 179 μ and under (nos. 21–32 in Table I) before another kind of setae were formed, with the exception of larvae nos. 24 and 28. They were equal in number on the two sides of the body, unless lost by accident or some other cause. The usual number appeared to be 4 per setal cone, although 5 had been observed in one larva (no. 24). In every setal cone the 4 or 5 setae were not of the same length and diameter, but were very regularly arranged in a definite order. The embryonic seta nearest the midline (no. 1 seta in Fig. 1 and Table I) was the second longest and second thickest among the four on each half of the body. The next one, occupying a more lateral position, was invariably the longest and the stoutest (no. 2 in Fig. 1 and Table I). The next one was the shortest and the thinnest (no. 3 in Fig. 1 and Table I). The outermost one (not shown in Fig. 1) was slightly longer and slightly thicker than the one medial to it. In larva no. 24 of Table I a fifth embryonic seta occurred outside the other four. This was the shortest and the finest in the larva.

Short spines were found on all the embryonic setae in approximately four irregular, longitudinal rows starting from a region about halfway between the base of the setae and the posterior edge of the shell (Fig. 1). Those on the coarser setae appeared to be stouter than those on the finer ones. They seemed to occur throughout the length of the setae.

The lengths of the different embryonic setae on both sides of the body were summarized in Table I. Due to the widely differing diameters of the first medial two, they could be distinguished from each other. The third and the fourth setae were, however, not easily distinguishable when removed from the larva. The diameter of each decreased very gradually from base to tip. The color was darker than that of other setae that appeared later. The embryonic setae were stiff and brittle. They broke off when they collided with hard objects on a slide. In older larvae they decreased in number and were absent in the oldest larvae.

The early larval setae appeared in an older larva during its early planktonic life. They occurred singly in widely separated regions along the mantle border. They were curved, stout setae, tapering rapidly in the last quarter or so to a sharp point. The stoutest among them was situated at each posterolateral region of the ventral shell valve and was equipped with stout spines. The maximum number of early larval setae seemed to be five on each side, although this was attained only on one side of larva no. 6. Of these, four issued from the ventral mantle, and one from the dorsal mantle. Many larvae had only 3 or fewer setae on each side. The stoutest seta was 300 to 350 μ long, the others, only half as long.

Larva no. 15 (Fig. 1) had a curved seta, morphologically indistinguishable from an early larval seta except in length, in diameter and in its point of origin. It was more slender and shorter. It issued not from the mantle border but from the setal cone. Whether it was produced during the embryonic or the larval stage is not known.

The late larval setae were the last to appear in the larva and were found only in the oldest larva (no. 1). In this there were about 24 of them evenly spaced in the lateral region of the mantle between the most anterior and the most posterior early larval setae. Finer and more flexible than these, they tapered gradually to a fine point.

Rotational movements of the dorsal shell valve were observed in a submerged larva lying with its ventral valve motionless at the bottom of a microscope slide. Protrusion and retraction of the lophophore, bending, extension and shortening of the individual cirri occurred now and again.

In swimming the anterior edges of the shell gaped open, the lophophore emerged from the shell and the tentacle and cirri spread out radially. The cilia on both the tentacle and cirri beat vigorously to drag the larva forward with the two bundles of primary setae trailing behind. On collision with another object the cilia immediately stopped beating and the larva sank. During forward progression the flat body regularly rotated about the anteroposterior axis and the two bundles of embryonic setae were sometimes parallel to each other and sometimes pointing outward from each other at an angle. On several occasions each bundle was simultaneously moved medially past each other to form a cross as the larva swam forward. The various positions of these setal bundles indicated a fine control by the muscles in the setal cone. While the animal was at rest, movements of individual embryonic setae in a bundle were also observed.

Movements of the early larval setae were also observed. These were capable of independent movements. The stoutest one on each posterolateral region was especially active and was seen to make intermittent lashing movements in the gape between the two shell valves. Whether these movements were an aid in swimming is not known. Movements of early larval setae increased in intensity and frequency when they collided with objects on the slide under observation in the microscope.

Discussion

The oldest larva in the present study resembled the discinid larvae described by Mueller (1860, 1861), Simroth (1897, Blochmann (1898) and Yatsu (1902). The youngest, however, though resembling the youngest discinid larva of Simroth in many ways, differed from it in the following respects. Firstly, Simroth's larva had 3 pairs of cirri instead of the 4 in the larvae of the present series. Simroth (1897, p. 4) believed that the larva with 3 pairs of cirri was in a stable state and that it would not develop additional cirri during its larval stage. Secondly, the innermost embryonic setae in Simroth's larva were bent. Thirdly, the longest embryonic seta in Simroth's larva no. 1 was the one next to the outermost, whereas in all the larvae examined in this study it was the one next to the innermost.

Presumably not realizing that the two discinid larvae he described could have been two different developmental stages in the ontogeny of a single species, Simroth (1897, p. 5) assigned them to two different species of discinids. However, the larvae in the present study consisted of a continuous series of growth stages, including those that are transitional stages between the two discinid larvae of Simroth. These stages included larvae that possessed both the embryonic and the early larval setae. The loss of the embryonic setae occurred in older larvae, thus transforming them into the more familiar so-called "Mueller's larvae." Obviously the larvae in the present study should be attributed to one single species of discinid. Their adults still remain to be discovered in the vicinity of Singapore. However, Blochmann (1898) relegated the 10 larvae from the Rhio Strait and those of Mueller (1860, 1861) to *Pelagodiscus atlanticus* (King).

The succession of three different types of setae in the brief span of discinid planktonic larval stage is a unique phenomenon unknown in brachiopod ontogeny. The present study showed the presence of two bunches of long embryonic setae on the lateral cones of the body wall in the youngest larvae in which the cirri were just mere short lobes of the lophophore. These lobes or short cirri of the lophophore were similar in appearance to those of the youngest planktonic larvae of *Lingula anatina* (Lamarck) the present author had observed and also to those of embryos of *Lingula* at the end of the embryonic period (Yatsu, 1902). Since the two bunches of embryonic setae were already present in the youngest larvae obtained, and also similar in length, width and appearance to those of older larvae, they were presumably embryonic structures elaborated from the substance stored in the fertilized ovum. The present work also showed the first appearance of the early larval setae in larvae of about 140 μ dorsal-valve length. These were obviously not normally present in younger larvae. These setae are called early larval setae, since they developed in early larval life. Another kind of setae, called late larval setae, appeared only in the oldest larva in the present study and in the larvae described by Mueller, Simroth, Blochmann, Yatsu, Eichler and Ashworth. These late larval setae, formed in late planktonic life of the larvae, prepared them for settlement to the sessile life of the postlarva. They reminded one of the setae that appeared near the end of the planktonic larval period of *Lingula* (Yatsu, 1902, Chuang, 1959). Simroth (1897, p. 15) regarded both the embryonic setae in his smaller larva and the early larval setae in his other larva as flotation aids. Presumably these two types of setae could also serve a protective function by making the larvae into less easily swallowed morsels. Moreover, they could be used in steering while afloat or drifting in a current. The rapid succession of three different types of setae is presumably an indication of their great necessity to these larvae. Mueller (1861) reported the resorption of the stoutest early larval setae and the loss of others in his discinid larvae that settled down to a sessile life in the laboratory. The present study (Table I) showed a gradual loss of not only the early larval setae in older larvae, thus confirming Mueller's observations, but also of the embryonic setae.

Simroth's larva no. 1 of 220 μ greatest diameter (1897, p. 3 and Tafel I, Fig. 1) was equivalent in size to no. 20 (Table I) of the present study, but in the development of setae it was in the same stage as larvae nos. 21 to 32 with the exception of nos. 24 and 28, which apparently had an earlier development of the early larval setae. His larva no. 2 of 420 μ diameter (p. 5 and Tafel I, Fig. 2) was equivalent in shell size to no. 5 but in setal development to no. 1 of the present study. Thus

Simroth's no. 1 was a discinid larva before the appearance of the early larval setae, while his no. 2 could well be an older larval stage of the same discinid species after the loss of the embryonic setae and the appearance of the next two types of larval setae, contrary to his (1897, p. 5) conclusion that the two larvae belonged to two different species of the same genus.

During the larval stage the shell valves changed in shape and proportion. In both shell valves there was a change from an almost circular plate of the youngest larvae to a pronounced oval with a greater transverse diameter. Next there was an ever-widening difference between the transverse and the longitudinal diameters of each shell valve during the last part of the larval life. Meanwhile the dorsal shell valve also increased in diameter much faster than the ventral. Finally there was a striking but gradual change in the posterior edge of the ventral shell valve from a convex to a concave one. This was presumably brought about by a finely adjusted modification of shell growth along the medial third of the posterior mantle edge. The formation of a bay at the posterior margin of the ventral shell valve, just as the formation of the pedicle, is a preparation for settlement to a sessile life. It is through this bay presumably that the pedicle is extruded for attachment to the substratum at the time of settlement.

Mueller (1860) reported the presence of a pair of statocysts in his discinid larvae. Although Blochmann (1898) denied their presence, they were observed by Yatsu (1902), Eichler (1911), Ashworth (1915) and the present writer.

A pair of eyespots, first reported by Mueller (1860), occurred only in the larvae found by Ashworth (1915). They were not observed by other writers.

If the sequence of appearance of organs during ontogeny can serve as an indication of their usefulness, then the pedicle, the statocysts and the late larval setae, which appeared towards the end of the larval stage, are presumably of little functional value to the larvae during the planktonic stage. They are presumably of use during settlement and the sessile life that follows.

In the ontogeny of *Glottidia* (Brooks, 1879) and *Lingula* (Yatsu, 1902; Chuang, 1959) shell size, as well as the number of pairs of cirri, increases during the planktonic larval life and either of them can be a fair index of the stage of larval development. In the ontogeny of a discinid, Table I showed that only shell size increased while the number of cirri remained constant at 4 pairs during the entire larval stage. Hence only shell size of a discinid larva could indicate the stage of development. Discinid larvae with 4 pairs of cirri need not necessarily be at the same stage of development, contrary to the belief of previous writers. In addition to shell size, the occurrence of organs or structures that develop at a definite stage during ontogeny may be a useful index of the age of the larvae. Such organs or structures include the different types of setae, the statocysts, the pedicle, the bay or notch on the ventral valve and the nephridia.

Mueller (1860), p. 79) rightly believed that his larvae were near the end of their larval life and on the way to adopting a sessile life. The absence of younger stages, he thought, was an indication that these had up till then remained in the shell of the adult. Ashworth (1915, p. 66), on the other hand, believed that the younger stages might live on the sea bottom and thus escaped capture. The occurrence of a series of developmental stages including the youngest larvae in the surface plankton of shallow water seems to indicate that the young stages can occur near the surface. The capture of these young and old stages near the shore also indicates the random movements of both young and old to or away from shore, contrary to the belief of Yatsu (1902, p. 107) that the larvae shortly before settlement made their way towards the coast.

Discinid larvae were obtained from February to April (Mueller, 1860, 1861) in the South Atlantic Ocean, from July to August or September (Yatsu, 1902), off Japan in the Northern Pacific and in October (Ashworth, 1915) in the northern part of the Indian Ocean. They were captured in the tropical West Pacific in July (Blochmann, 1898) and in June, August, September and November by the present author. This indicates the restriction of breeding to the summer and autumn. More intensive collection may possibly extend the breeding season in tropical waters.

SUMMARY

1. Thirty-three discinid larvae were obtained one meter below the water surface in the daytime in the shallow part of the Johore Strait, north of Singapore.

2. The oldest among them, 462 μ in longitudinal diameter, resembled the "Mueller's larva" discovered by Mueller and referred to *Pelagodiscus atlanticus* (King) by many subsequent authors.

3. The youngest among them, 108 μ long and 115 μ wide in dorsal shell, resembled Simroth's smaller discinid larva of 220 μ diameter in many essential features.

4. The larvae in this study showed a gradual transition in many morphological characters, indicating that they were a series of developmental stages in the ontogeny of a single discinid species.

5. The two bunches of long embryonic setae were gradually replaced by the curved early larval setae in the medium-sized specimens. Towards the close of the larval stage the third kind of setae appeared from the mantle border.

6. The dorsal shell valve maintained its oval shape during the entire larval stage. The posterior edge of the ventral one, however, gradually changed from convex to concave, passing through a stage when it was almost a straight line.

7. The statocysts and the pedicle appeared in older larvae.

8. The number of cirri remained constant throughout the larval stage.

9. The larvae were compared with the discinid larvae previously recorded. Their developmental changes and the breeding season of discinids were discussed.

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REPRODUCTIVE BEHAVIOR IN THE SPIDER CRAB, LIBINIA EMARGINATA (L.) [1]

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Crustaceans have complex behavior patterns. Among these are patterns concerned with reproduction and more specifically mating (reviewed by Schöne, 1961). Mating behavior patterns vary among different species and in a number depend upon the physiological condition of the female for their initiation. For example, in some species, mating is correlated with the pre-adult molt of the female and the transferred sperm are stored in the seminal receptacles of the female. Among the portunids, the male protects the pre-molt female and mating occurs immediately after she molts. This behavior is initiated by a pheromone released into the water by the pre-molt female (Ryan, 1966). In some brachyurans (*e.g., Callinectes, Carcinus*), mating is apparently dependent on a condition of ripeness of the ovary (Chidester, 1911; Broekhuysen, 1937). Among Majidae, Schöne (1968) reported that *Maja verrucosa* males protect the females beneath them prior to mating. In some brachyurans submissiveness of the female when seized is necessary for mating to occur (Chidester, 1911). Finally, the mating posture varies among species.

The initiation and probably other aspects of the mating behavior in these crustaceans evidently require rather specific communications between potential mates. In land forms this is often visual and auditory (Salmon and Atsaides, 1968) while in aquatic forms it may also be chemotactic. Chemotatic communication would simply involve one or more pheromones as in the one clear-cut example described by Ryan (1966). In view of the interest in mechanisms of communication in Crustacea as well as other organisms it seemed of interest to report observations on mating behavior of the spider crab, *Libinia emarginata* (L.), and to describe for the first time a heretofore unrecognized male-female association, here called "obstetrical behavior."

MATERIAL AND METHODS

From June to early September, 1967, male and female *Libinia emarginata* obtained from the Marine Biological Laboratory Supply Department, were kept in an aquarium (approximately 3′ × 5′ × 9″) through which sea water constantly flowed. This arrangement was initially selected to provide storage for the crabs for another purpose. However, as specific behavior patterns began to be recognized, immature animals were removed and only mature animals (5–6 males and 8–14 females) were retained. Routine feeding (*Mytilus* and *Spisula*) was initiated, females were marked with insoluble dyes for individual identification, and their brood chambers were examined periodically. The crabs were observed for reproductive behavior at intervals during most of the day and evening.

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OBSERVATIONS

Ovigerous females were observed from early June until early September. The females are apparently able to produce at least 3–4 consecutive broods during the period studied. In these crabs spermatozoa enclosed in spermatophores are transferred to the seminal receptacle of the female at mating. At the time of oviposition, the eggs pass the seminal receptacle where they can interact with spermatozoa, and are then discharged into the "brood chamber" where the egg mass becomes attached to the pleopods. The last known date of oviposition in this study was September 1. As has been reported by Costello *et al.* (1957), the eggs at the time of oviposition are a bright orange-red in color. The color changes from orange red to brown as development proceeds in the egg mass. Zoeae were apparent within the egg chorion a few days prior to hatching. In the case of at least six marked females development to the swimming zoea was completed in 25 days. In nine of thirteen

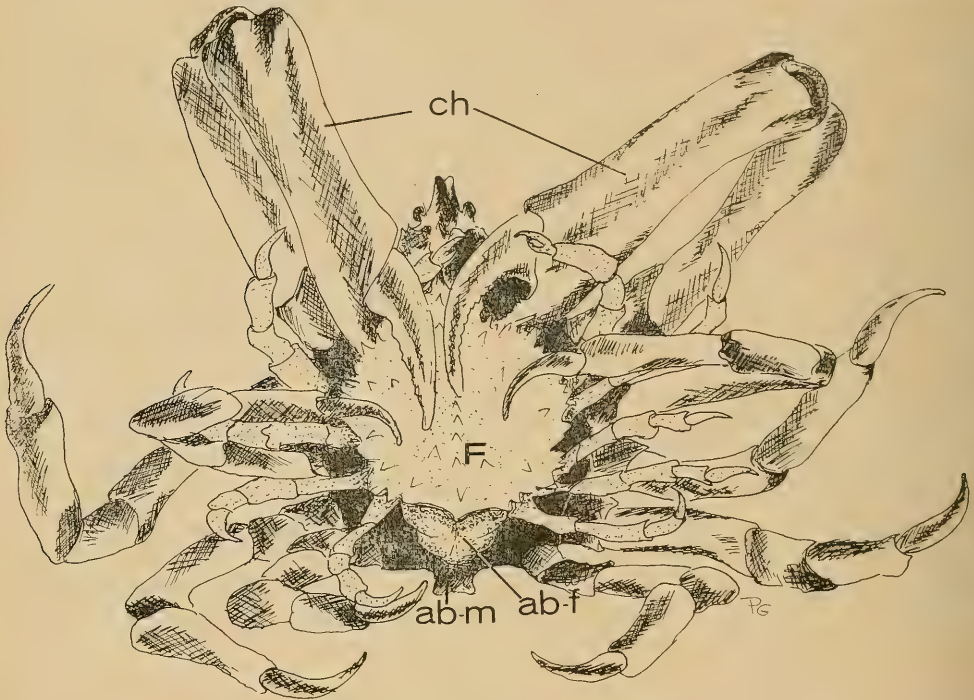

FIGURE 1. Mating position. Chelae (ch) of the male are bent as he holds the female (F) in the rostral region and rotates onto his carapace. Abdomens of male (ab–m) and female (ab–f) are extended.

recorded cases, the females had completed oviposition of a new brood in less than 12 hours after releasing zoeae. In four other cases, oviposition occurred after more than 12 hours following zoea release.

Two definite and distinct types of behavior were associated with the release of the zoeae, *i.e.,* mating and obstetrical behavior.

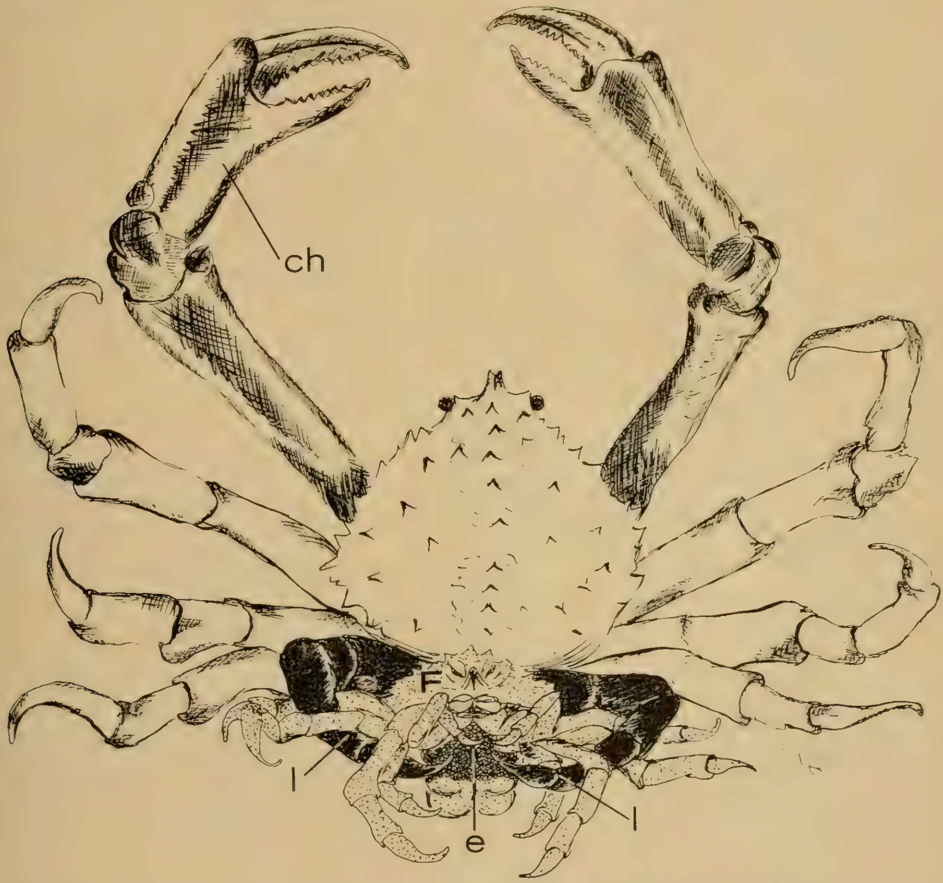

FIGURE 2. Obstetrical position. Female (F) held at right angles to body of the male. The fifth pair of walking legs of the male (l) are inserted into the brood pouch filled with eggs (e) containing zoeae about to hatch. Chelae (ch) of the male are extended at this time.

Mating

In the aquarium containing females about to release zoeae, the males can be observed walking about on the tips of their dactyls. They appear to be actively searching. Encounters at this time with non-gravid females or females which have either just released their larvae or are about to release larvae generally result in mating. Females with eggs in the early stages of development were never observed to mate.

In six recorded cases, the males first grasped the legs of the female with his chelae and positioned her beneath him. His chelae were bent in the manner shown in Figure 1 as he grasped the female in the rostral region. The abdomen of the female was pulled down and bent back at the same time the male's abdomen was lowered. Copulation then began and the male frequently rotated over onto his carapace holding the female above him. Once copulation had been completed, the male resumed a walking position, released his hold on the head of the female, once again grasped her by the legs and released her.

Obstetrical behavior

Immediately preceding the release of zoeae by a female, males were seen actively searching. Males were very aggressive at this time and frequently fought with each other. In sixteen recorded cases, when a female about to release zoeae was encountered, the male captured her and placed her beneath him. Two males might fight for possession of a female and sometimes lose her to a third male. Eventually, a male grasped the female by her legs with his chelae, moved her posteriorly, and positioned her body at right angles to his own. The tips of his fifth walking legs were inserted beneath the abdominal flap to hold her in this position (Fig. 2). The male then frequently backed into a protected area and stood with chelae outstretched. In this position he would fight with other mature males or drive away immature males or females. While being held in this position, the female fanned her abdomen and zoeae were released. Following zoea release she cleaned the remaining egg cases from her pleopods with her chelae. When she had finished the cleaning operation the male released her. Juvenile males and females as well as females with broods at early stages of development were never held in such a position by the male.

Discussion

Libinia emarginata is abundant in coastal waters of the north Atlantic. Bumpus (1898a, b) reported the collection of breeding animals from May through early August while Thompson (1899) collected larvae as late as September 4. Costello *et al.* (1957) stated that development from oviposition to release of larvae takes about one month. In the present study, development was completed in 25 days and the last observed date of oviposition was September 1.

In *Libinia emarginata* there seems to be little sexual display other than the searching walk of the male. Mating position seems similar to that observed in other brachyurans (see review, Schöne, 1961). Although copulation occurs only in newly molted females in many crustaceans, mating in adult *Libinia* occurs between broods and is not associated with molting. Whether the female first mates at the time of the final molt to adulthood is not known. Mating between broods is apparently not needed to produce viable embryos. In *Menippe mercenaria* many broods are produced between intermolts and the sperm are carried over at molting (Cheung, 1968). In *Libinia emarginata* females have been observed to mate with more than one male, suggesting that storage of sperm from several males is possible.

Chemical or mechanical stimuli have been implicated in reproductive behavior in many arthropods. The presence of a pheronome in the portunids has been established (Ryan, 1966). In *Libinia,* the predictability of the onset of mating and obstetrical behavior, the active searching by the males, the increased fighting among males and the selectivity of the animals to be placed in the obstetrical position suggests that the behavior patterns are initiated by some chemotactic substance, *i.e.*, pheromone(s). Direct experimental evidence for the presence of a pheromone has not yet been obtained. Submissiveness on the part of the female in *Libinia emarginata* may be necessary for mating and for the obstetrical behavior.

Assuming the action of a pheromone(s), it must either come from the female or be released by or with the zoeae at hatching. Production of such a substance and initiation of the behavior patterns seem to be independent of time of day since they occurred at all times of day and night although with greater frequency during the evening hours. In the laboratory situation this could be influenced by artificial conditions such as the fluorescent lighting or crowding. However, predictability, selectivity of participants and the fact that behavioral patterns are not interrupted by feeding or grooming activities suggest that this behavior may occur in nature.

What evolutionary advantages are there in the development of such behavioral patterns? The obstetrical behavior may have evolved to protect the female as the zoeae are being released although females are able to release zoeae without the presence of a male. In *Maja,* the male protects the female beneath him while the zoeae are released, (Schöne, 1968). *Libinia emarginata* males have been observed standing over and protecting females which had only two or three legs. They seemed unable to place such females in the obstetrical position. The distinctiveness of the behavior in *Libinia* may in part be accounted for by the great differences in size between male and female.

Among Majidae, both *Libinia emarginata* and *Maja* have a new egg mass in the brood pouch a short time after zoea release. In *Maja,* the crabs mate prior to zoea release. In *Libinia* it seems possible for a female to produce a new egg mass without mating again although mating frequently occurred either before or after zoea release. Production of a pheromone at this time may insure mating before the next brood. Among Majidae where no further molts occur after the adult stage has been attained, mating at the time of zoea release may insure carryover of sperm from one season to the next.

SUMMARY

1. Data concerning reproductive behavior and development in the spider crab, *Libinia emarginata* (L.) are recorded. Ovigerous females were collected from early June to early September. Females are apparently able to produce at least 3–4 consecutive broods of 25 days development each during a breeding season.

2. Two distinct reproductive behavior patterns are associated with the release of zoea on the 25th day of development, *i.e.,* mating and obstetrical behavior. These male-female associations are stereotyped behaviors. The position of the bodies of the male and female differ in the two behavior patterns.

3. The predictability of onset of these behaviors and the specificity of the patterns suggest their possible initiation by pheromone(s).

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THE OCCURRENCE OF DINOFLAGELLATE LUMINESCENCE AT WOODS HOLE[1]

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The use of sensitive photomultiplier radiometers has brought about recognition of the very widespread occurrence of bioluminescence in the oceans. Flashing has been recorded in nearly all parts of the oceans and in almost all locations studied (Clarke and Wertheim, 1956; Clarke and Kelly, 1965). At night using proper equipment luminescence may be recorded continuously in the upper layers of the ocean, usually within the euphotic zone and above the thermocline (Clarke and Kelly, 1965; Yentsch, Backus and Wing, 1964). However, the organisms responsible for the near-surface luminescence have been definitely identified only in exceptional circumstances such as during unialgal blooms (Harvey, 1952) or in very eutrophic embayments such as Phosphorescent Bay in Puerto Rico (Clarke and Breslau, 1960; Seliger *et al.,* 1962).

Many authors have suggested but not shown that the organisms responsible for this common near-surface luminescence are the dinoflagellates, very common phytoplankters with carbohydrate cell walls and two flagella, one encircling the cell at the midline and the other directed posteriorly (Clarke and Kelly, 1965; Harvey, 1952; Kelly and Katona, 1966; Schiller, 1937). The work reported here is one of three studies conducted under diverse conditions and at diverse locations to determine the relationship between the occurrence of luminescent dinoflagellates and the common oceanic bioluminescence.

In the work described here the dinoflagellates and bioluminescence present in Woods Hole Harbor between November, 1965, and November, 1966, were studied. The species capable of luminescence were identified, their concentrations in the water were measured throughout the year, and the amounts of luminescence in the water were determined at least once a week by testing samples brought into the laboratory darkroom. The populations were also tested for susceptibility to light inhibition of luminescence and for the presence of an endogenous diurnal control of the amount of luminescence produced.

In a preliminary study made during August and September, 1965 (Kelly and Katona, 1966), water was brought into the laboratory at Woods Hole Oceanographic Institution and the luminescence in the water was measured. The species of armored dinoflagellates capable of luminescence were identified and although cell concentrations were not measured, these organisms appeared to be present in sufficient numbers to explain the luminescence in the water. In addition, the populations displayed an endogenous rhythm in luminescence and in sensitivity to light inhibition of luminescence. It became apparent that a study over an extended period of time at one location was required to show if luminescent dinoflagellates are usually present in sufficient numbers to explain the amounts of luminescence recorded in the water, and whether endogenous diurnal rhythms of luminescence are present throughout the year.

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LUMINESCENCE OF ISOLATED CELLS

Individual dinoflagellates were tested to determine which species were capable of contributing to the luminescence recorded in whole water samples. Cells were isolated by micropipette into 0.5 ml. of water and stimulated in front of a photomultiplier radiometer. Methods were described in detail by Kelly and Katona (1966). The results are given in Table I. Kelly and Katona (1966) and Sweeney (1963) listed other dinoflagellate species known to be luminescent. All species showed similar intensity (approximately 10^{-4} μW per cm.^{2} at 10 cm. from the sensing surface) and duration of flashing. Occasionally a prolonged flash was recorded, lasting as long as 3–4 seconds, and the specimen could never be recovered afterward, probably because it splashed against and adhered to the side of the

TABLE I

Dinoflagellates examined for flashing. Numbers refer to months in which tested, from 8, 65 to 8, 66. One to ten specimens were tested on each date given

Species	Luminescent		Fluorescent		Photosynthetic pigments
	yes	no	yes	no	
Ceratium fusus	8, 65; 9; 10				always
C. lineatum		9; 10; 11; 12; 1		12	always
C. tripos		9; 10; 11			always
Glenodinium lenticula		9; 10; 11; 3			never
Gonyaulax digitale	9; 8, 66				always
G. spinifera	8, 65				always
Gymnodinium splendens		10			always
Peridinium spp.	varies				varies
P. claudicans	8; 10	12; 8, 66		12; 8, 66	never
P. conicum	6; 10		9, 66		never
P. depressum	4				never
P. divergens	11		12		never
P. globulus	10; 11				never
P. granii					?
P. leonis	9; 10; 11; 8, 66				never
P. mite		8, 65			?
P. oceanicum	9; 10				never
P. oblongum		8, 66		8, 66	never
P. pallidum	9; 12		12; 8, 66		never
P. pellucidum		4			never
P. pentagonum	8, 66		8, 66		never
P. punctulatum	9; 8, 66		8, 66		never
P. subinerme	11; 12; 1; 2; 3		2		never

sample tube. Usually, however the flash duration did not exceed 10 milliseconds. Specimens of *Peridinium subinerme* and *Peridinium pallidum* remained alive and capable of flashing for as long as 7 to 21 days while isolated in the dark in test tubes without any food other than what little might be in the 0.5-ml. Millipore-filtered sea water that contained them. None of the cells divided, although culture medium and microalgal suspensions were added to several of the samples.

Some of the species were tested for UV fluorescence under an American Optical Co. fluorescence microscope (see Table I). Long-wavelength UV produced only the typical ruby-colored fluorescence of chlorophyll-*a* (in diatoms and *Ceratium lineatum,* the only photosynthetic dinoflagellate examined). Short-wavelength UV produced a continuous blue-green fluorescence in all the luminescent dinoflagellates examined. Fluorescence originated in cytoplasmic particles ranging in size from less than 0.001 mm. to 0.003 mm. Nine species (3 or more specimens of each) were examined in this way (see Table I). All specimens of the 6 luminescent species showed the blue-green fluorescence while the 3 non-luminescent species did not. The long duration of the fluorescence indicated that the organisms were not merely stimulated to luminesce by the UV irradiation.

The fluorescence passed through a blue-green 480 nM. wavelength interference filter without appreciable visible attenuation but was attenuated by 470 and 490 nM. filters. The fluorescence therefore peaks quite sharply at or near 480 nM., which is the luminescence peak for several species. Eckert (1966) reported the "microsources" of luminescence in *Noctiluca scintillans* fluoresced in this range.

It seems likely that the blue-green fluorescence is an indication of luminescent capability. The peak at 480 nM. suggests that the fluorescence is due to the chemical that is the source of luminescence. This is not surprising since the luminescent compound must be easily excited to an active state and it should be expected that light output from this excited molecule would have similar spectral composition whether the excited state is produced by an enzymatic reaction or by UV irradiation. It also seems that luminescence probably originates in the fluorescent cytoplasmic particles, as it does in *Noctiluca scintillans.* Several other organisms such as copepods (Clarke *et al.*, 1962) and *Cypridina* (Harvey, 1952) have fluorescent luminescent organs.

The bioluminescence of several dinoflagellate species merits further discussion: *Ceratium fusus* is notable for being the only species of *Ceratium* that has been definitely shown to be luminescent (Sweeney, 1963; Kelly and Katona, 1966). Nordli (1957) maintained luminescent cultures of this species, but they were lost and he was unable to culture luminescent forms again. In the present work few of the cells tested were luminescent. This suggests that only certain cells are luminescent and that luminescence is an inherited characteristic. An inheritable ability for luminescence is also suggested by the existence of both luminescent and non-luminescent clonal strains of *Noctiluca scintillans. Ceratium tripos* has been reported by several authors to be luminescent (Harvey, 1952) but it was not so in Woods Hole and Nordli's (1957) cultures of this species were not luminescent. This places the earlier reports in doubt, although there is the possibility that both luminescent and non-luminescent forms exist as in *Ceratium fusus.* The luminescence of *Peridinium claudicans* was also variable; it was luminescent during certain months and not others in Woods Hole. The taxonomy of *P. claudicans*, however, is confused and more than one species may have been included under this name.

TABLE II

Summary of flashing rates (flashes per 30 seconds) and luminescent cell concentrations (cells per liter) during 1965–1966 at Woods Hole

	Dec.				Jan.				Feb.			Mar.				Apr.
	7	15	21	27	3	12	25	31	8	15	23	2	16	23	29	6
1. Mean	160	76	100	76	84	111	190	223	117	189	110	176	144	149	146	152
2. Maximum	179	94	128	94	109	186	208	241	159	238	181	195	198	162	165	185
3. Minimum	141	56	66	65	61	163	200	81	138	74	160	58	137	130	121	144
4. Mean, 1100–1400 hrs.	169	74	117	77	97	76	177	221	99	164	96	189	—	143	146	134
5. Mean, 2000–2300 hrs.	158	89	79	79	74	158	199	215	148	197	138	177	167	156	136	173
6. Luminescent cells	71	68	42	21	54	141	243	892	602	993	446	640	237	118	157	52

	Apr.			May				June				July				Aug.			Sept.			Oct.
	12	20	27	4	10	17	31	7	16	21	28	5	13	16	27	3	13	30	7	13	28	11
1.	179	94	150	96	112	152	136	66	65	8	31	19	50	2	10	41	25	34	83	217	199	137
2.	196	117	208	107	152	205	164	84	89	18	40	26	78	5	17	87	41	45	104	260	206	177
3.	78	78	115	84	93	119	81	49	44	1	10	15	32	0	5	12	3	8	62	201	189	90
4.	175	98	180	98	135	173	—	70	66	—	34	18	38	3	16	67	33	—	85	196	199	—
5.	194	93	124	98	97	—	157	64	66	—	40	22	29	—	9	18	7	42	89	250	—	158
6.	70	23	35	26	51	11	45	13	12	1	2	8	40	0	40	172	160	292	20	33	29	54

VARIATION OF LUMINESCENCE IN WATER SAMPLES

Fifteen-liter surface water samples were brought into the laboratory darkroom from near the harbor dock of the Woods Hole Oceanographic Institution. Dates of sampling are shown in Figure 1 and are given in Kelly (1968); luminescence stimulated by bubbling air through the water was measured about every week throughout the year. The method and equipment were described by Kelly and Katona (1966). All measurements were performed in a darkroom at sea-surface temperature. For consistency the equipment and procedures were carefully kept the same throughout the entire program. Samples were collected after dark the day before measurements commenced around midday. Measurements were taken every hour to detect any endogenous diurnal rhythms that might have been present (see Kelly and Katona, 1966). Observations were also made on the effects of light inhibition of luminescence. They are described below.

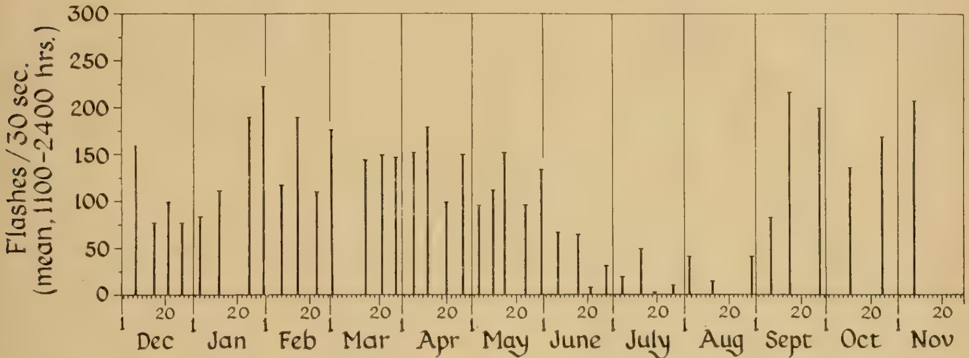


FIGURE 1. Bioluminescence recorded in water samples from Woods Hole Harbor during 1965 and 1966.

The amounts of luminescence found throughout the year are shown in Table II and Figure 1. These are compared below to the numbers of luminescent cells found in the water. The detailed measurements were too numerous to report here but are given by Kelly (1968). Surprisingly no significant endogenous rhythms were found. As shown in Table II, the averages of the flashing rates during midday and after dark were similar: 139.6 and 136.8 flashes per 30 seconds, respectively, through the end of May. These averages suggest a lack of any significant endogenous day-night rhythms in flashing. There was usually some variation within any day's observations as shown in the examples in Figure 2. However, the variation was usually random, and it has been impossible to determine any cause for it. There were increases in flashing rates after dark on the 12th and 25th of January and the 8th and 23rd of February, but these were probably fortuitous since they were not repeatable. Figure 2 shows typical daily measurements from November through May plotted against time of day. The curve marked by ▲ (from Kelly and Katona, 1966) is typical of the endogenous rhythm found in August and September, 1965. The one marked by △ shows the measurements of January 12, 1966, which were the most suggestive of an endogenous rhythm during 1966. The one shown by ○, taken January 25, 1966, is more typical and shows no rhythm. The last curve, ●, is a plot of the means and its lack of variation indicates that hourly variations on any given date were random.

The endogenous rhythms found by Kelly and Katona (1966) in August, 1965, were thus not present during the rest of the year. The genus *Gonyaulax* was dominant during the August, 1965, studies, but luminescent forms of the genus were not often found during the rest of the year. Also, *Gonyaulax* is the only genus reported to have an endogenous luminescence rhythm in culture. It would appear therefore that endogenous diurnal rhythms of luminescence are uncommon insofar as the situation in Woods Hole is typical.

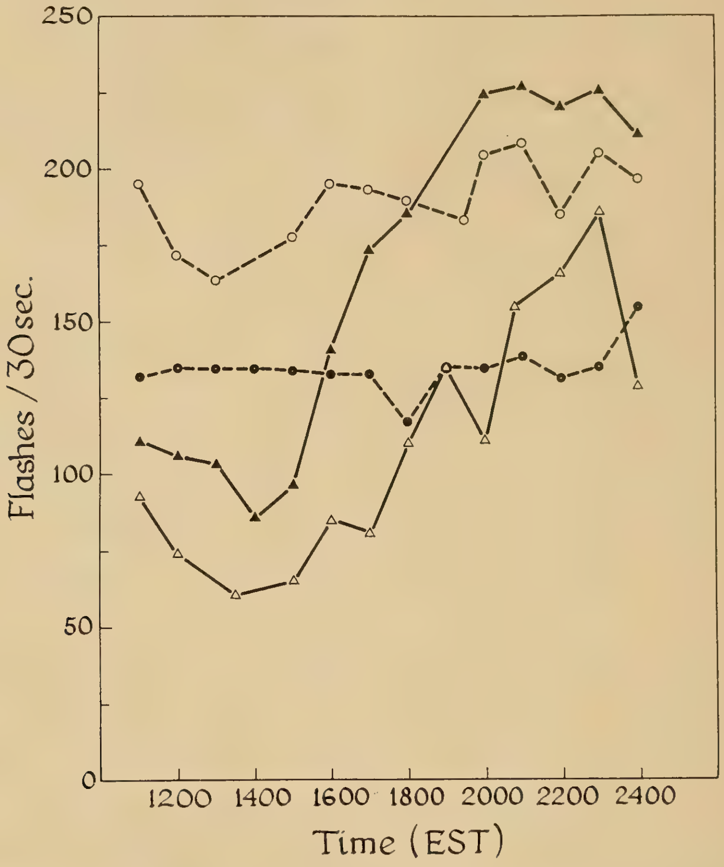


FIGURE 2. Luminescent flashing in samples of water from Woods Hole Harbor measured from noon to midnight on various dates. See text for explanation.

Numerous observations were made that indicated dinoflagellate flashing is nearly always inhibited by light. This would explain the daytime decreases in luminescence usually recorded in the natural environment (Clarke and Kelly, 1965; Kelly and Katona, 1966). Kelly and Katona (1966) found light inhibition of flashing as well as an endogenous diurnal rhythm in sensitivity to that inhibition

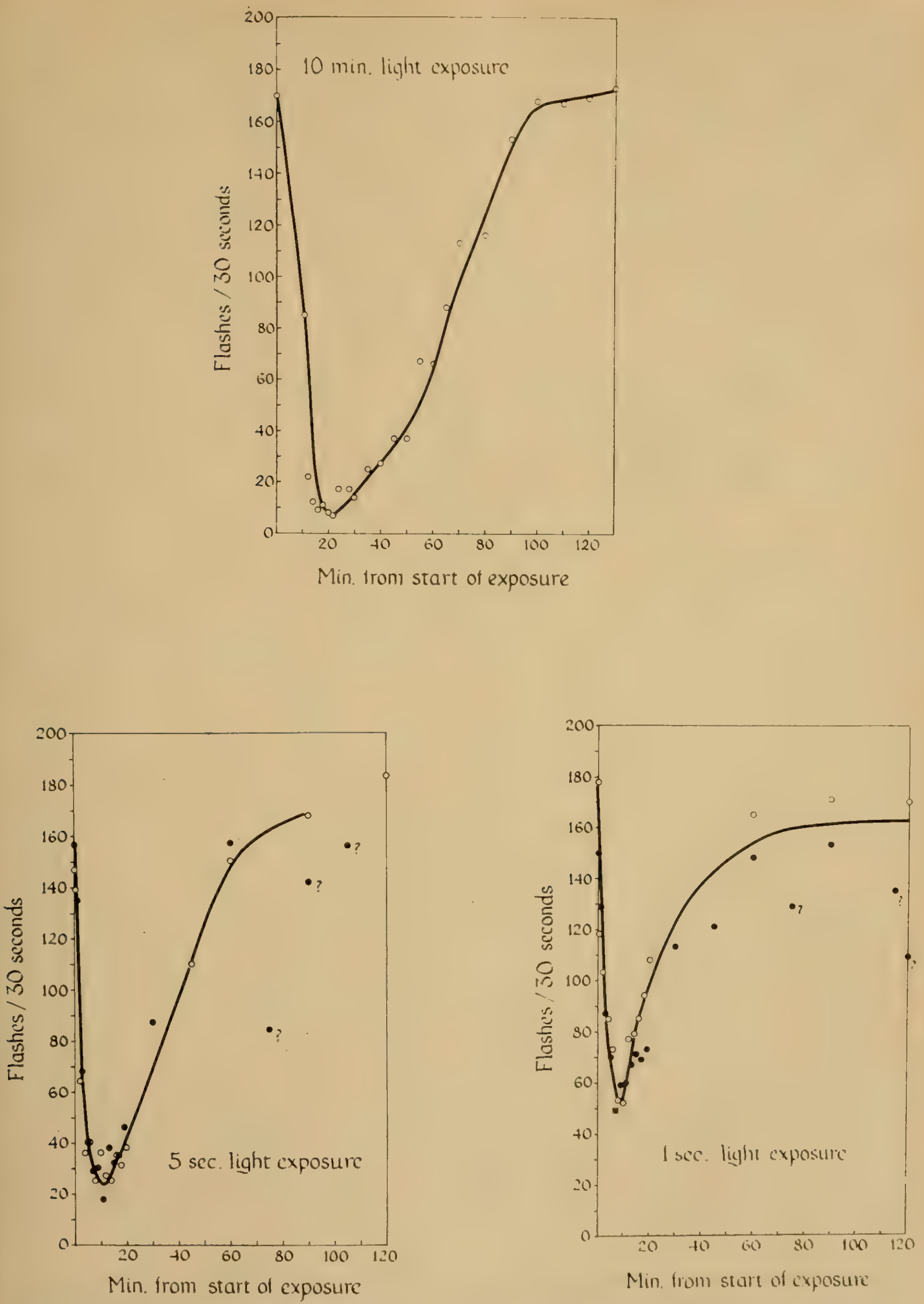


FIGURE 3. Light inhibition of luminescence in water collected from Woods Hole Harbor. See text for details.

during August, 1965. In the subsequent work casual observations of flashing inhibition were made at least once a month by turning on the lights in the darkroom (350 lux at the water surface, G.E. "cool white" bulbs, approximately 4100° K color temperature) at the end of a day's observations and measuring the effect on flashing rate. Inhibition was always found, although the amount of inhibition varied greatly.

Light inhibition of luminescence was quantitatively measured on April 13, 1966, in water collected on April 11 and kept continuously in the dark. Results are shown in Figure 3. The water was exposed to the room lights (350 lux as above) for periods of 10 minutes, 5 seconds, and 1 second. All of these exposures gave considerable inhibition. In the 5-second and 1-second observations two barrels of water were used simultaneously (the white and black circles in Figure 3). Recovery was always complete within 100 minutes of the start of exposure. Interestingly, minimal flashing rates were found 10 minutes after the end of exposure in all cases, including during the 10-minute observation, *i.e.*, inhibition appears to commence when the lights go on. An explanation of this must await better understanding of the physiology of these organisms.

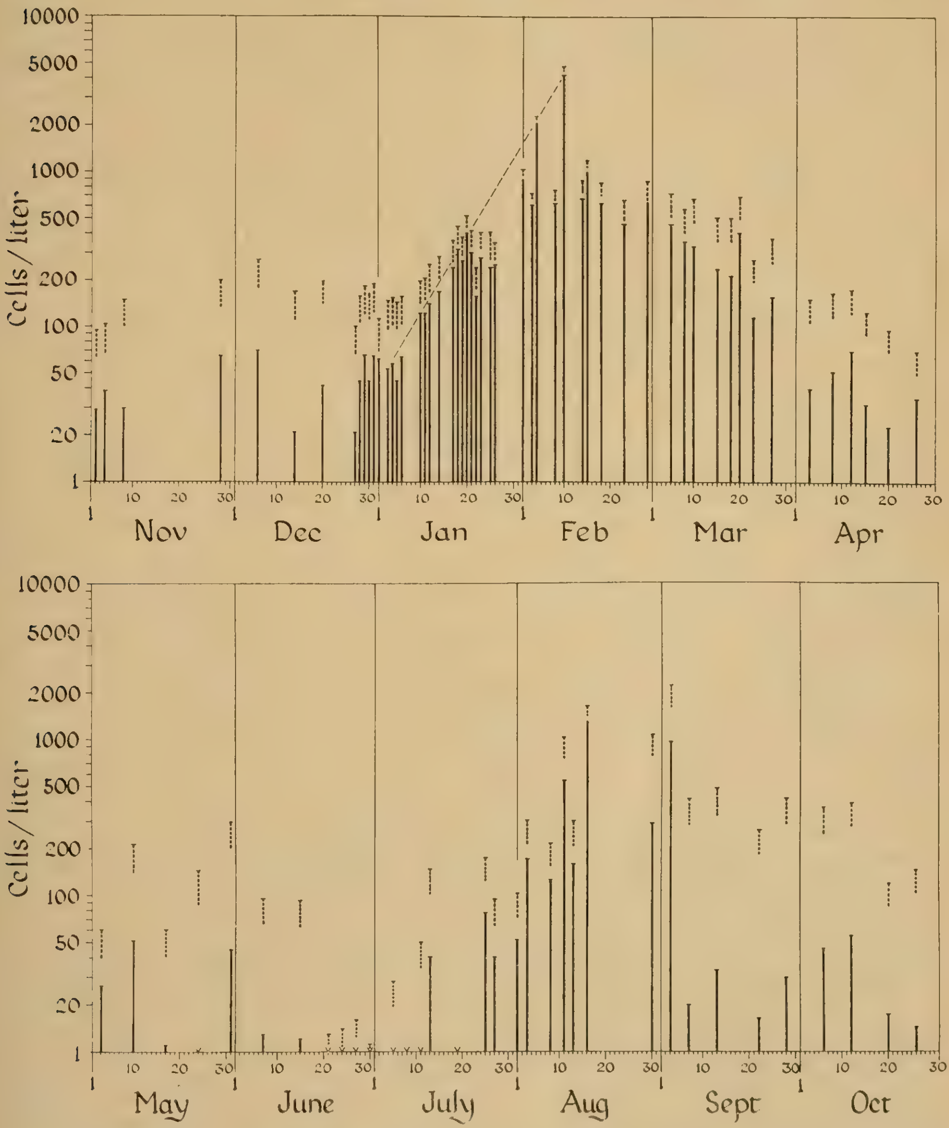
VARIATION OF DINOFLAGELLATE POPULATIONS

It was necessary to know the concentrations of the luminescent cells so as to determine if sufficient numbers were present to account for the recorded luminescence. Since very few cells per liter may produce measurable quantities of luminescence, and since usual methods of determining phytoplankton populations depend on small (usually 200-ml.) water samples (Braarud, 1958), it was necessary to use a new counting technique.

A quantitative 10-liter water sample was taken from the Woods Hole Oceanographic Institution dock and a surface tow was taken for taxonomic use with a 0.035-mm. mesh aperture, 20 cm. diameter net. Dates of sampling are shown in Figures 4 and 5. The 10-liter quantitative samples were passed through a 400-mesh (0.035-mm. mesh aperture) stainless steel screen. The retained material was very carefully washed from the screen with 15 to 25 ml. of Millipore-filtered sea water from a squeeze bottle and the concentrated samples were preserved with Rhode's iodine or with formalin. The samples were allowed to settle for at least 24 hours, the supernatant was removed, the residue was transferred with washing into a 10-ml. graduated conical centrifuge tube, and the samples were again allowed to settle. The supernatant was again removed, the sample was transferred to a watchglass, and the centrifuge tube was rinsed with sufficient water to bring the sample volume to 1 ml. The sample was strongly agitated in the watch glass and a 0.1-ml. aliquot was transferred to a microscope slide using a hypodermic syringe. The syringe was rotated continuously to prevent the organisms from settling in the barrel during transfer. The dinoflagellates on the slide were tabulated and counted by species under the 10-power objective of a compound microscope. A second or third 0.1-ml. aliquot was examined if necessary to count enough organisms. Since the organisms in 10 liters were concentrated into 1 ml., 0.1 ml. of the concentrate contained the organisms in 1 liter of the original sea water.

Specimens from the net tows were drawn and identified. The taxonomy is discussed by Kelly (1968) and will be published elsewhere. Although immediate sight identification of dinoflagellates is difficult, it was possible to recognize the species in a sample during counting if detailed studies of the two samples were made first.

There were possible sources of error in the sampling technique but with care all proved negligible. Sample losses during transfer and during removal of the super-



FIGURES 4 and 5. Dinoflagellate concentrations found in samples of water from Woods Hole Harbor during 1965 and 1966. Dashed lines: total dinoflagellates. Solid lines: luminescent species. The straight dashed line points out an apparent logarithmic increase in populations that were nearly all *Peridinium subinerme*.

natant were not significant since examination of the supernatants, glassware, and screen very rarely showed specimens left behind. Inconsistencies due to transfer to the slide and measurement of the sample aliquots were negligible since results were repeatable (see Table III). Similarly, these repeatable results argue against inconsistencies in the original sampling and screening. Some cells may have passed through the screen; indeed, Wheldon (1939) found considerable loss of specimens when collecting on 0.065-mm. aperture mesh bolting silk. However, many species are smaller than 0.065 mm., but few are smaller than the 0.035-mm. aperture mesh used here. Since no armored dinoflagellates could be found on cleared Millipore filters through which screened water was passed, and since water passed through the screen rarely showed bioluminescence, loss through the screen must have been usually insignificant.

TABLE III

Dinoflagellate concentrations in cells per liter measured at various times of day in Woods Hole Harbor on November 29, 1965

Species	Time			
	1530	1730	2100	2300
Ceratium fusus	10	8	9	8
C. lineatum	77	78	74	84
C. macroceros	4	5	5	5
C. tripos	38	36	39	28
Glenodinium lenticula	13	17	17	23
Gonyaulax spp.	1	1	1	1
Peridinium claudicans	3	6	6	4
P. divergens	3	3	2	2
P. leonis	3	3	2	2
P. oceanicum	2	2	1	1
P. ovatum	6	5	4	6
P. pallidum	37	36	33	37
P. subinerme	2	2	3	3
Phalacroma spp.	1	1	1	1
Total of all species	199	203	196	204
Total of luminescent species	64	66	61	63
Liters counted	2	2	2	2

Possible sources of error are misidentification of species of small size or confusing shape and normal random sampling errors. Misidentification was rare, and was insignificant in the overall results. Extra aliquots were counted so as to increase the sample size when the specimens were few enough to give significant random counting errors.

The concentrations of total armored dinoflagellates and of luminescent dinoflagellates are shown in Table II and in Figures 4 and 5. Thirty-two species were present of which 16 were luminescent. The species are listed in Table I; their concentrations are given in Kelly (1968). Maximal numbers of dinoflagellates occurred during January, February, and March, and during August and September, with decreases in between. The concentrations are compared below with the amounts of bioluminescence recorded. Variations in cell concentrations during a single day (November 29, 1965) are given in Table III. The uniformity of these results shows a lack of tidal and diurnal fluctuation in populations in Woods Hole Harbor and verifies the reproducibility in the counting method.

CORRELATION OF LUMINESCENCE WITH DINOFLAGELLATE CONCENTRATIONS

The regression lines of luminescent dinoflagellate concentrations and flashes recorded per 30 seconds were calculated and are plotted in Figure 6.

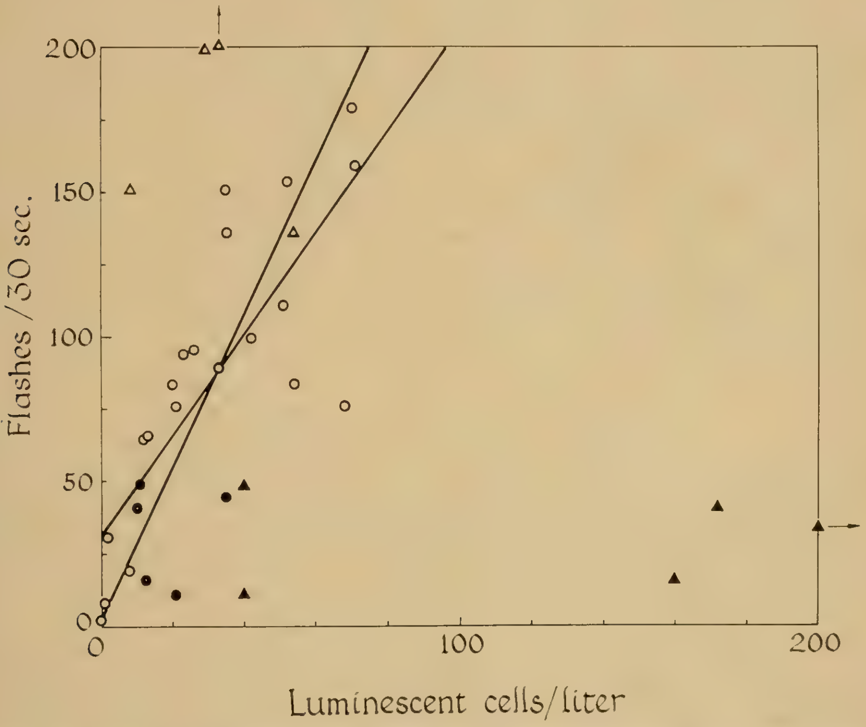


FIGURE 6. Correlations of dinoflagellate concentrations and luminescence in samples from Woods Hole Harbor during 1965 and 1966. See text for details.

On a few occasions described below the data were obviously invalid and not included in the calculations. The high flashing rates on May 17 and between September 13 and October 11 were due to luminescent cells too small to be collected and counted and hence were deleted from the correlations. They are designated by △ in Figure 6. The luminescence of the small cells was realized because luminescence was recorded in water that passed through the collecting screen. Flashing in the screened water varied from 53 to 103 flashes per 30 seconds compared to 136 to 217 flashes per 30 seconds in the unscreened water. However, the flashing rates in the screened water were probably not closely related to the number of luminescent small cells originally present since some may have been retained on the screen and many that passed through were probably damaged. A liter of water collected on September 15 was passed through the screen and centrifuged. Many diverse microflagellates were collected, but it was impossible to determine which were responsible for the luminescence.

The flashing rates from July 13 and from July 27 through August 30 (designated by ▲) were low for the number of apparently luminescent cells counted. It was at first feared this discrepancy might be due to some failure in technique such as contamination of the samples with toxic materials, but visual observation while swimming off the dock at night showed qualitatively less luminescence than would have been expected from the populations of *Gonyaulax* spp. counted in the samples. If flashing rates for those dates are plotted against luminescent cell counts not including *Gonyaulax* spp. they fall within the expected range (● in Figure 6). The data from those days were not used in the regression calculations and it seems likely that the *Gonyaulax* spp. populations were not luminescent. This was realized after the populations had declined, too late to directly test the luminescence of the cells.

As the number of flashes increased they tended to overlap on the records and thus at higher flashing rates many of the flashes could not be counted. Those luminescence records could not be expected to have a direct relationship with the number of luminescent cells present. Because of this, samples with more than 100 cells per liter were not included in the regression calculations.

The equations of the regression lines shown in Figure 6 are as follows:

$$Y = (1.75 \pm 0.88)X + (31.5 \pm 21.2) \qquad (1)$$

$$X = (0.38 \pm 0.68)Y + (-0.5 \pm 35.5) \qquad (2)$$

X and Y represent luminescent cells per liter and mean flashes per 30 seconds, respectively. The errors are given for a 90% confidence interval. The calculations show a correlation coefficient of 0.81 between flashing rates and cell concentration, which with this sample size assures that there is less than a 0.001 probability that there is no correlation.

It is impossible to give a simple ratio relationship between luminescent dinoflagellate population density and flashing rates because of the scatter in the regression curves. This scatter indicates that factors other than simple population density influenced the amount of flashing recorded, which is not surprising considering the diverse environmental conditions and species studied. Species may differ in the number of cells capable of luminescence. For example, not all specimens of *Ceratium fusus* were capable of flashing, while nearly all of *Peridinium subinerme* were. Similarly a particular species may differ in its ability to luminesce under different environmental conditions, *i.e.,* the ability may be influenced by available energy, by nutrient concentrations, or by temperature. Also, different species or a particular species under different environmental conditions might differ in threshold to stimulus, and hence the constant stimulus used in the experiments might more readily produce flashing in some populations than in others.

It is nonetheless apparent that there was a strong relationship between the amount of flashing and the density of the luminescent populations present in the water. Except in the few instances when small protozoa were mainly responsible for the luminescence, armored dinoflagellates were present in sufficient numbers to explain the flashing recorded.

DISCUSSION

From this work it may be concluded that for the period studied dinoflagellates were the only major cause of bioluminescence in Woods Hole Harbor, except on rare occasions when microflagellates were present that were too small to be taken on the collecting screen. The question arises whether the armored dinoflagellates are able to account for the luminescence that is found almost everywhere in the surface regions of the oceans. Extensive studies at other locations (Kelly, 1968; to be reported separately) indicate that dinoflagellates are responsible for luminescence in many diverse oceanic environments. Dinoflagellates are common organisms (Lebour, 1925; Schiller, 1937) and are evidently commonly luminescent.

The question also arises whether dinoflagellate luminescence has evolved separately in several diverse dinoflagellate genera, or whether the luminescent species are derived from a common ancestor. Because of the diverse biochemical systems of luminescence in other marine organisms such as euphausids, ostracods, shrimp, and bacteria (Harvey, 1952; Johnson and Haneda, 1966) it seems likely that bioluminescence evolved several times in the oceanic environment. This argues a strong selective pressure for bioluminescence in the sea. Several features of dinoflagellate luminescence, however, argue that the systems are biochemically and hence genetically related.

The four species whose spectral outputs have been studied have similar emission curves (Fig. 7). *Pyrodinium bahamense, Gonyaulax polyedra,* and *Pyrocystis lunula,* very diverse species, all have a spectral peak between 470 and 480 nM., rising sharply from between 450 and 435 nM. and dropping off by 550 nM. (Taylor *et al.,* 1966; Hastings and Sweeney, 1958; Swift and Taylor, 1967). *Noctiluca scintillans* has a similar light output but its spectrum was not as accurately measured (Nicol, 1958). The spectral differences between these species are very slight and can probably be attributed to differences in technique. Although the emission curves of other organisms peak near this wavelength, they usually have a different shape (Johnson and Haneda, 1966).

The very similar emission spectra of widely diverse dinoflagellate species argue for the same light-producing enzyme-substrate reaction throughout the group since the bonding energies and structure of the light-producing molecules determine their output spectra. In addition, Hastings and Bode (1961) found that cross-reactions of *Gonyaulax monilata* and *G. polyedra* luciferin and luciferase produced light, as did cross-reactions between *G. polyedra* and *N. scintillans* (Hastings, personal communication).

Dinoflagellate luminescence spectra are similar to sea-water light transmission curves and this suggests an adaptation of luminescent dinoflagellates to the environment: an evolutionary adaptation on the molecular level. Light transmission of most offshore sea water is at a maximum between 470 and 480 nM., *i.e.,* daylight at depths below about 10 or 20 M. is blue (Jerlov and Koczy, 1951). The luminescence and maximal visual sensitivity of other marine organisms are usually in a similar color range (Nicol, 1958) although the biochemical mechanisms of their luminescence certainly differ. Although McElroy and Seliger (1962) suggest a purely biochemical function, these spectral similarities seem to suggest an adaptation of the luminescence of marine organisms to the blue maximum transparency of sea water or to the blue sensitivity of marine animals' eyes.

The question is thus raised as to whether the identical emission spectra of dinoflagellates are due to inheritance without change from a highly adapted parent species, or whether the spectra arose independently through parallel adaptation to identical environmental characteristics. The cross-reactions between the species and the fact that the spectra of these species show more similarity to each other than to unrelated marine forms suggest very similar or identical chemical structures and derivation from a single parent stock. This may well have been a primitive gymnodinioid form similar to the swarm cells of *Noctiluca scintillans.* The retention by these forms of the genetic information allowing them to luminesce at the expense of energy utilization indicates that luminescence probably continues to serve some adaptive function. Further study of luminescence may have con-

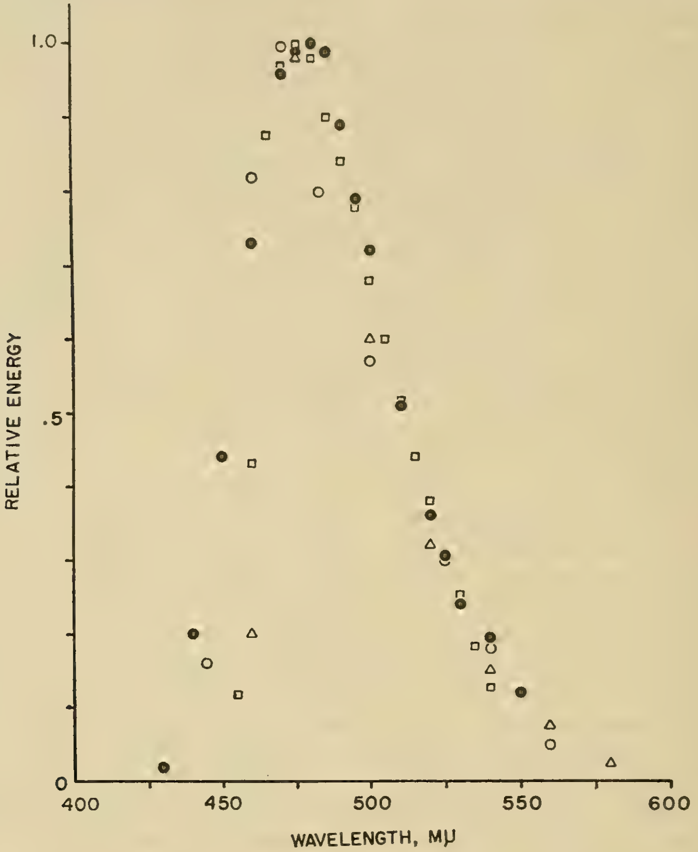


FIGURE 7. Emission spectra of luminescent dinoflagellates. ○ *Noctiluca scintillans* (Nicol, 1958) ; □ *Pyrodinium bahamense* (Taylor *et al.*, 1966) ; ● *Gonyaulax polyedra* acetone extract (Hastings and Sweeney, 1957) ; △ *Pyrocystis lunula* "cysts" (Swift and Taylor, 1967).

siderable bearing on our understanding of both the phylogeny of dinoflagellates and the adaptive values of luminescence.

If the ecology of luminescence in the ocean is to be understood, we must be able to predict the spatial and temporal distribution of dinoflagellates and must understand their luminescence characteristics. This means that much more work is needed on the trophic dynamics of phytoplankton in the ocean, on the biogeography of dinoflagellates, on their taxonomy, and particularly on their characteristics in culture. To date only a very few luminescent species have been cultured, and the only common colorless species included in these is *Noctiluca scintillans*. It thus appears that an understanding of the characteristics and distribution of luminescence must await better understanding of other fields of marine biology.

Because of the diverse species of luminescent dinoflagellates and because of their diverse characteristics and adaptations, I greatly doubt that any single model will serve to predict or describe the distribution of luminescence. Rather, the problem will require considerable descriptive understanding of the variation of species distributions, of photoinhibition of luminescence, of endogenous rhythms, of the luminescence abilities of different species, of the energy sources for those species and of the relationships between their distribution and the environment. Such an understanding will be slow in coming and will depend on a number of diverse fields of study. I believe, however, that the present work shows dinoflagellate luminescence to be very common and to deserve attention from workers in many fields.

I would like to thank Dr. George L. Clarke for encouragement and constructive criticism and for providing necessary facilities. I would also like to thank Drs. Johan Hellebust and David Wall and Mr. Barry Dale for frequent help and suggestions. Mr. Paul Willis made numerous measurements while I was away from Woods Hole.

SUMMARY

1. Bioluminescence in the water in Woods Hole was measured once a week from November, 1965, to November, 1966, and was related to the occurrence of armored luminescent dinoflagellates.

2. Thirty-two species of dinoflagellates were identified, 23 of these were tested for luminescence as isolated cells. Sixteen species were found to be luminescent.

3. Population densities of dinoflagellate species were measured by counting individuals screened from water samples. The concentrations of luminescent species varied from nearly 1000 cells per liter in February, 1966, to less than 5 cells per liter during June and July, while total dinoflagellates ranged from more than 4000 cells per liter to 5 cells per liter.

4. Amounts of luminescence radiometrically recorded in air-bubble-stimulated water samples correlated with the numbers of luminescent armored dinoflagellates present. Exceptions to this correlation were found when population densities were too high for the radiometric recording techniques to be accurate and during May and August, 1966, when the luminescent organisms were too small to be collected and counted.

5. Endogenous diurnal rhythms of flashing were found only during August, 1965 (Kelly and Katona, 1966).

6. Light inhibition of flashing was found whenever it was tested (at least once each month), although sensitivity to light inhibition varied greatly—as little as 1 second of exposure to 350 lux illumination reduced flashing by $\frac{2}{3}$.

7. The ecology and evolution of luminescent dinoflagellates are discussed. It is suggested that dinoflagellates are responsible for most near-surface luminescence, that luminescent forms are derived from a single common ancestor, that the ability serves an adaptive function, as yet undetermined, and that much more work on a number of areas will be required before a detailed ecological understanding of dinoflagellate luminescence is obtained.

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THE GENERAL ECOLOGY AND GROWTH OF A SOLITARY ASCIDIAN, CORELLA WILLMERIANA[1]

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The diverse assemblage of marine organisms growing on boat hulls, floating docks and other man-made installations is commonly termed the fouling community. Many practical investigations into the development and control of these communities have been undertaken (see Anon., 1952, for review). Ecological study of the fouling communities has been hampered, however, by the lack of information on the life cycles, growth and survival rates, and other properties of the individual species forming this complex (Grave, 1933; Berrill, 1950; Abbott, 1957; Moore, 1958).

One group which may form an important, even dominant, part of the fouling complex, but which has received very little attention, is the Ascidiacea. Scattered ecological data on this group can be found in a few papers, for example those by Huntsman (1921) on *Chelyosoma,* and Just (1934), Hŭus (1937), and Millar (1953) on *Ciona.* Although certain aspects of ascidian population ecology have been studied by Millar (1952, 1954) and Goodbody (1961a, 1961b, 1962, 1963, 1965), a detailed study of the growth of many individuals of a single population is lacking.

Of the thirteen species of solitary ascidians that occur in the Puget Sound region of the Pacific Northwest coast of the United States, one of the more common occurring on marina floats is *Corella willmeriana* Herdman (which will hereafter be referred to as *Corella*). This small (2–3 cm. in height), fairly transparent animal breeds throughout the year. The eggs are shed into the atrial chamber, where they are fertilized and develop into the free-swimming tadpole stage before being released. The aims of this study were to determine the growth rate of the young post-larval stages of *Corella* in the field, the approximate size at sexual maturity and the average life span. Observations on predation and on interspecific competition for space have been included, since it is the relative interplay between all these factors that determines the establishment, persistence, and general success of any species population characteristic of fouling communities.

Material and Methods

Study area and apparatus

Field observations were carried out at the Bremerton Yacht Club on Phinney Bay, adjacent to the city of Bremerton, Washington, from April 24, 1966, to April 15, 1967. These floats, although composed of such diverse materials as creosoted and fiberglass-covered styrofoam, wood, and rubber (old airplane fuel tanks), harbor a luxuriant growth of marine animals, including usually a large population of *Corella*. In order to study the growth rate and settling preferences of the young post-larval stages of *Corella*, two frames of polyvinyl chloride were constructed (Fig. 1). Each frame held 32 8.3 × 10.2 cm. glass plates 1 mm. in thickness, arranged on four sides of the frame, four pairs of plates per side. The plates were suspended vertically in pairs so that organisms could settle on only one surface of each plate. The frames (designated A and B) were suspended about 24 cm. below the water surface by nylon rope and plastic-coated wire from the walkway on the floats.

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[1] From a thesis submitted in partial fulfillment of the requirements for the M.S. degree, University of Washington, 1967.

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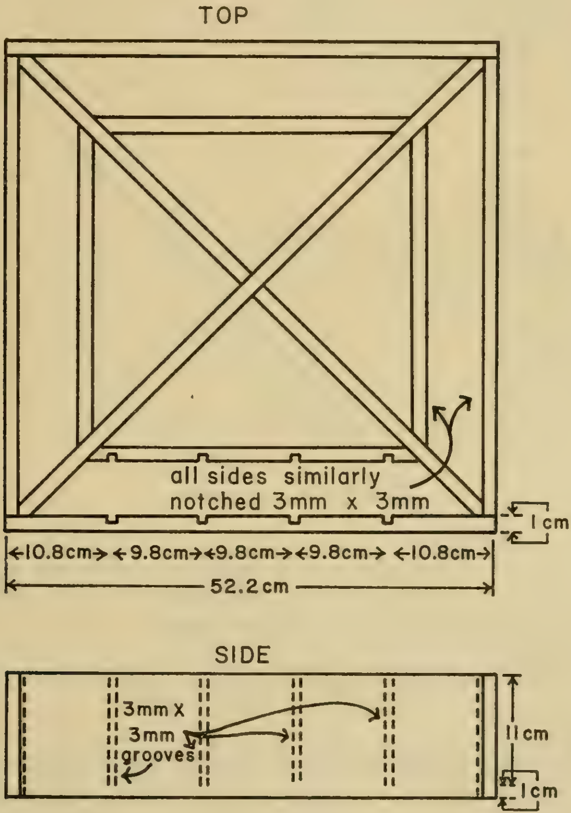

FIGURE 1. Diagram of settling frame.

On April 23–24, 1966, a large number of new creosoted styrofoam floats were placed in the water at the Bremerton Yacht Club by the management. Since this was the date on which the two frames were initially submerged, a good opportunity was provided for comparing each month the extent of fouling of the floats and frames. It was hoped that this would give some indication of the effect (or lack of effect) of different substrates on settlement intensity and pattern.

Replacement schedule for the glass plates

A record of the first month's growth of the newly settled *Corella* was obtained for each month of the year by replacing monthly four plates from each frame. For submergence times of 2–11 months, two plates were removed from each frame each month. The remaining eight plates in each frame were left in the water for 12 months. The schedule of the particular plates to be replaced was decided before the frames were placed in the environment by using a random numbers table.

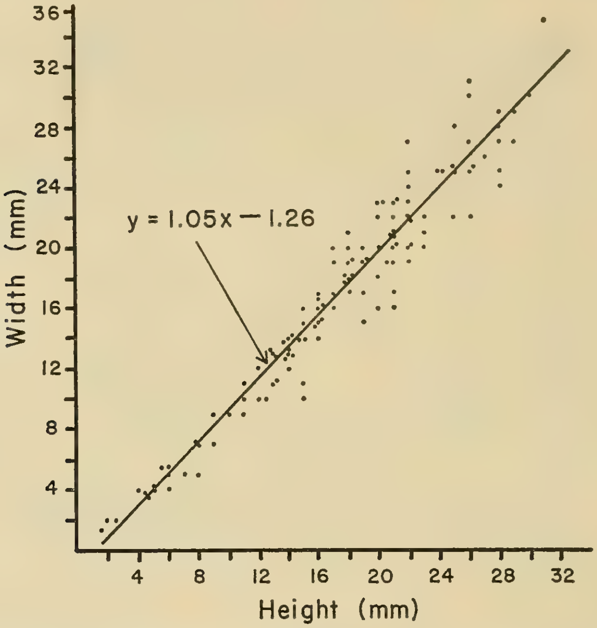

FIGURE 2. The relationship of height to width of 124 *Corella* from natural populations. The line was fitted by linear regression.

Pomerat and Weiss (1946) indicated that the surface texture of the substrate is an important determinant of the species and numbers of animals that will settle. In order to determine to some extent whether the tadpoles preferred to settle on smooth or roughened glass, four of the eight plates replaced each month had a smooth surface exposed for settlement, as did eight of the sixteen plates left in the frames for the entire year. The remaining plates were roughened on one side with coarse carborundum paper.

On 8/20/66 it was discovered that both the nylon rope and plastic coated wire supporting frame A had been cut. The frame was retrieved from the bottom a month later and after cleaning, a new set of glass plates was installed and the frame submerged on 9/25/66. The schedule of replacement of the glass plates was begun again from the beginning, but time did not permit an additional year of study of frame A from the date of its resubmergence. Thus only from April through July was settlement on the two frames comparable.

As each plate was removed from its frame it was immediately isolated in a clear water-filled plastic sandwich box. These were transported back to the laboratory in an ice chest and examined with a binocular dissecting microscope without ever being removed from the boxes. In this way the organisms which had settled on the plates were disturbed as little as possible, and if anything was dislodged, it was kept with that plate. During the replacement of plates and examination of the frames each month, the frames were raised to the surface of the water but were never removed from the water, thus minimizing any undue disturbance and possible tearing off of organisms. The water in which the frames were suspended was fairly sheltered, and tidal currents, though present, were not swift. Therefore, many *Corella* and other organisms were able to remain tenuously attached to the frame.

Method of size measurement of Corella

The measure of individual size used in the growth studies was determined as follows. The height of *Corella* shows a linear relationship (Fig. 2) to its width, as measured across the two siphons. Therefore, although both height and width are a valid measure of size, the latter was used because it was easier to make and entailed a minimum of disturbance to the animals. Newly metamorphosed *Corella* possesses paired atrial siphons (which later fuse to form one atrial siphon) and a single branchial siphon. For newly metamorphosed individuals, the width was measured from the branchial siphon across one of the atrial siphons.

Results and Discussion

General description of changes occurring on settling frames

The two settling frames were placed in the water on April 24, 1966. During the first two months of submergence, large clumps of filamentous diatoms, hydroids, and barnacles developed. By June 19 a few specimens of *Corella* were large enough to be seen easily on the frames, the largest measuring 4.5 mm. in width. After three months' submergence the number of *Corella* had increased greatly; the largest were 12 mm. in size and were sexually mature, as evidenced by the full sperm duct in all the largest individuals and the presence of a few eggs floating in the atrial chamber of some. Large masses of hydroids (mostly *Obelia* spp.) and the colonial ascidian *Distaplia occidentalis* were also present, though the number of barnacles and filamentous diatoms had declined. In August, frame A was missing, but *Corella* was very abundant on frame B, and the largest individuals, now four months old, measured 30 mm., a size near maximum for float populations of this species. *Distaplia* colonies were numerous, and the colonies of another colonial tunicate, *Diplosoma macdonaldi* (formerly *D. pizoni*) had begun to appear.

At the September sampling, most of the hydroids on frame B had disappeared, as had most of the largest *Corella*. Some empty entire and torn tests were found still attached to the frame and to some of the glass plates submerged since April. The cause of death of most of the *Corella* is not known. It was most likely not environmentally caused, because hundreds of 1–2-month-old *Corella* on the frame and plates appeared perfectly normal and healthy. A small percentage of the mortality was caused by flatworms of the genus *Eurylepta;* this will be discussed later.

Very few sexually mature specimens of *Corella* were observed on frame B in October; most of the population measured about 1 cm. in width or less. The largest of these had become sexually mature by the end of November, and throughout December, January, and February large clumps of mature *Corella* dominated the surface of frame B and those plates which had been in the water for 4–6 months. Very little growth of *Distaplia* occurred during the winter months; most of the individuals had disappeared or had died back to an apparently quiescent base. On the other hand, colonies of *Diplosoma* grew rapidly between December and March, covering most of the bare space (including a large part of the plates replaced each month) and overgrowing the *Corella* to some extent. Between November and February, when large numbers of *Corella* tadpoles were settling on the clean plates each month, many specimens 300 μ or less in size were found beneath the spreading *Diplosoma* colonies. The overgrown individuals near the edge of the colonies were often still alive, but those near the center of the colonies had died. Figure 3 shows three tracings made of a glass plate removed from frame B and suspended in running sea water in the laboratory for two months. The *Diplosoma* has eventually grown around the *Corella* without growing over them; in all cases there is a space surrounding the *Corella.* These events are similar to those which occurred on the floats and frame B at Bremerton; *Diplosoma* usually grew around rather than over organisms larger than 1 cm. in height.

By March a few of the largest *Corella* had disappeared from frame B, especially those nearest the top edge of the frame and plates. This may have been due to low surface salinity, caused by the heavy rainfall during the winter months which resulted in a surface layer of fresh water that could be seen floating over and mixing to some extent with the salt water underneath. At the completion of these observations in April, 1967, nearly all the adult *Corella* had disappeared from frame B. This second wave of mortality is believed to be due to the large masses of filamentous diatoms that had accumulated on frame B starting in March and increasing tremendously in April. Thick mats of the diatoms were found attached to the siphonal end of the remaining adult *Corella,* and in some the branchial basket was clogged to a variable extent. Large sheets of *Diplosoma* were still present, and *Distaplia* had reappeared. Hydroid growth was negligible.

The changes occurring on the creosoted floats were quite similar to those occurring on frame B, with a few exceptions. When initially submerged, in April of 1966, barnacles settled thickly on the floats (nearly all of these fell off a few months later), but relatively few settled on the frames and virtually none on the glass plates. Most of those that did settle on the frames had fallen off after three months. Large populations of *Corella* colonized both the frames and the new floats during the summer of 1966, but after the large adult mortality in September, younger individuals did not replace them so rapidly or completely on the floats as on frame B. During the winter months the anemone *Metridium senile* became very dense on the floats and grew tall enough to extend beyond the *Corella* present there. On frame B, in contrast, specimens of *Corella* were always larger and more numerous than *Metridium.* It appears that the smooth surface of the polyvinyl chloride frames provided a substrate somewhat more suitable for *Corella* than for some of the other species present in the area.

After frame A was resubmerged in September, filamentous diatoms and hydroids were the first to appear, though not so thickly or in so short a time as in the spring. The diatoms were gone by November, although the hydroids (mainly *Obelia* spp.) continued to increase in numbers and length steadily until April, when the project was terminated. Throughout the winter months large parts of the frame and plates were covered by broad flat colonies of *Diplosoma*. *Corella* was not observed on frame A or its plates until January. In February about three or four mature individuals 2–2.5 cm. in size were observed, suggesting that there were a few young unnoticed *Corella* present before January. Even in March only about a dozen were observed, and most of them had not yet reached reproductive size. By April these were mature and healthy-looking. Almost

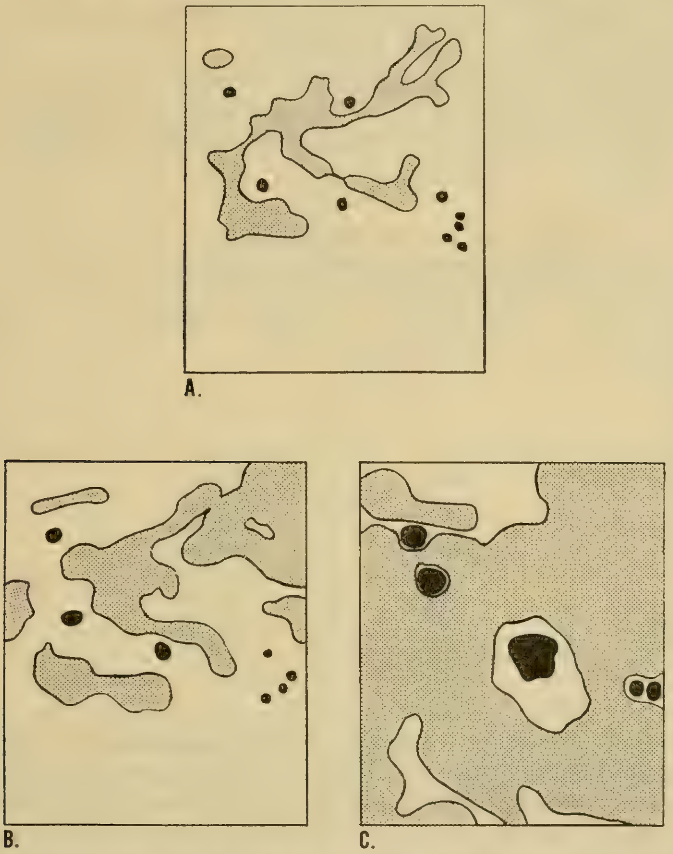

FIGURE 3. Diagram showing growth of *Corella* (solid black circles) and the colonial ascidian *Diplosoma macdonaldi* (stippled) on a glass plate in the laboratory. Unutilized space is blank. A, 8/23/66; B, 9/6/66; C, 10/29/66.

no filamentous diatoms had accumulated, possibly because frame A was in a shadier location and subject to faster water currents than frame B. Large numbers of laminarians and *Agarum* sp. were found growing on the upper edges of frame A in March, and although most of them were removed so as not to cover the frame completely, they had grown back again in April; several had reached a length of one meter. *Distaplia* colonies appeared in November but grew very little until the beginning of March. By the end of April the colonies had increased in size, and at the termination of observations the most abundant organisms on frame A were *Diplosoma, Distaplia, Corella* and *Obelia.*

It is obvious that an important difference between the two frames developed after the resubmergence of frame A. Before that there had been some quantitative differences, but in general they were comparable. During the winter months, however, while frame B contained a large breeding population of *Corella,* frame A remained nearly completely free of *Corella.* Floats nearby frame A possessed at least a few *Corella,* so theoretically there should have been tadpoles in the plankton. Table I presents the total number of *Corella* settling on the plates removed monthly from frame B, and it will be seen that not until a breeding population had become established (July–August) on the frames did there appear large numbers of tadpoles settling on the plates. After the first adult mortality in September, only a few tadpoles settled in October. But beginning in November, after a second breeding generation had developed, there were again large numbers of tadpoles settling. When the second wave of adult mortality occurred in the spring of 1967, the number of tadpoles settling decreased sharply, in spite of the fact that the *Corella* on frame A and some of the marina floats were still alive and healthy. Thus it seems to be extremely local recruitment of tadpoles that accounts for the presence of nearly all of the individuals on frame B. Nair (1962) has made a similar observation for compound ascidians in his extensive paper on marine fouling.

TABLE I

Surface water temperature and size after one month of growth of the largest 10% of Corella at the Bremerton Yacht Club (April 1966–April 1967)

Date	Total no. settled	Mean size of largest 10%	H_2O temp. (° C.)
April 24	—	—	11
May 21	0	—	12
June 18	1	1.4 mm.	15
July 16	0	—	16
Aug. 20	71	1.7 mm.	17
Sept. 25	1164	1.9 mm.	14.5
Oct. 22	10	1.5 mm.	11.5
Nov. 20	604	.356 mm.	11
Dec. 18	439	.500 mm.	9.5
Jan. 22	525	.507 mm.	8
Feb. 19	284	.402 mm.	8
March 19	89	.394 mm.	8
April 15	6	.430 mm.	10

Seasonal fluctuations in settlement and growth rate of young Corella

Because of the small size of the plates and the fact that frame A had to be restarted, the variation on plates from frame B submerged for longer than one month was too great to permit any realistic evaluation of the growth rate of *Corella* older than one month. Fair numbers of tadpoles settled on the plates which were replaced monthly, although this number fluctuated widely from month to month (Table I).

In agreement with Pomerat and Weiss (1946), there was a significant difference between the numbers of individuals settling on smooth and roughened plates each month. For the eight months considered appropriate for making these paired observations, 662 settled on smooth and 1405 on roughened plates. The hypothesis of random settlement was rejected by χ^2 ($p << 0.01$), indicating that *Corella* either preferentially settled on or adhered to the roughened surfaces. Between plates of different surface texture, no significant difference in individual size of the largest 10% settling each month could be demonstrated, by an analysis of variance. Therefore, size measurements from all four one-month plates from frame B were pooled each month. Only the largest 10% were used, based on a statement by Moore (1958) that such a measure of the few largest should give a reasonable indication of growth for the entire period.

TABLE II

Number of newly settled Corella 200 μ in size or less on plates submerged for varying lengths of time

Month of analysis	Duration of submergence	Number of *Corella*
Nov.	1 mo.	549
	7 mo.	0
Dec.	1 mo.	200
	2 mo.	48
	4 mo.	4
	8 mo.	0
Jan.	1 mo.	169
	3 mo.	14
	9 mo.	5

One might question the validity of assuming that the monthly size measurements actually represent in most cases an entire month's growth. Many projects have been carried out to determine the normal sequence in fouling of denuded or clean surfaces submerged in the sea (Scheer, 1945; Anon., 1952). Although this problem is far from being solved, it does appear that certain types of organisms settle first (usually bacteria, diatoms and suctorians) and other organisms settle later, even if ready-to-settle larval stages have been present in the water for the entire time. *Corella* tadpoles, however, apparently prefer to settle on bare surfaces, as evidenced by the number of newly settled tadpoles observed on plates submerged for varying lengths of time (Table II). (Similar data have been collected on a larger scale by Goodbody (1965) for *Ascidia nigra,* which also shows a marked preference for unfouled surfaces.) The indications are, then, that *Corella* will settle immediately on clean surfaces such as glass, supporting the assumption that the largest specimens represent a full month's growth. The close relationship between size of the breeding population on the frames and number of young *Corella* on the one-month plates is further support for this assumption. It might have been better to submerge panels on which tadpoles had been allowed to settle in the laboratory and for which the exact age was thus known, but this was unfeasible for the present study and would have introduced other problems of analysis.

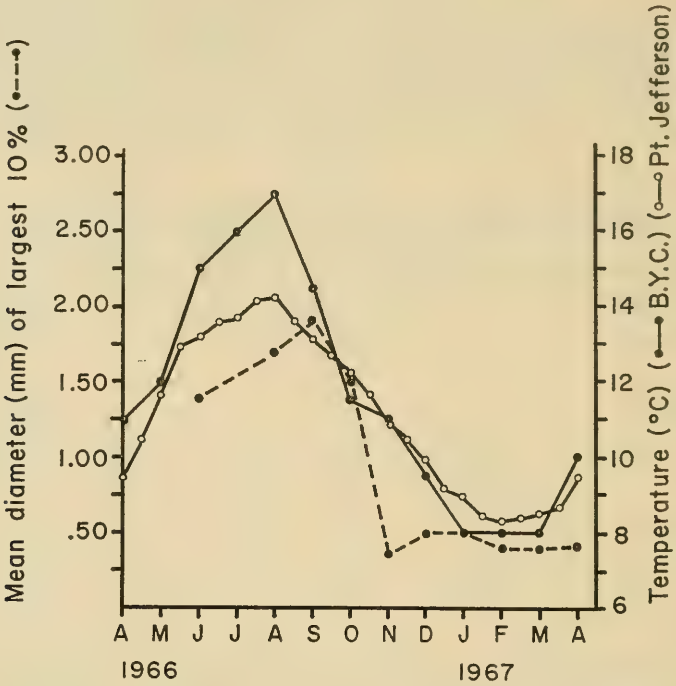

FIGURE 4. A comparison of surface water temperatures at Pt. Jefferson and the Bremerton Yacht Club with the size attained by *Corella* after one month of growth.

In order to determine whether there were seasonal fluctuations in the growth rate of the young stages of *Corella,* the average size attained in one month by the largest 10% of the newly settled individuals for each month throughout the year was measured (Table I). It should be noted that some of the figures are more reliable than others, being based on larger sample sizes. For instance, in October very few mature individuals were present on frame B, and a total of only ten *Corella* was counted on all four of the one-month plates removed that month. In order to obtain a reasonable size value for such a small sample, the size given (1.5 mm.) is an average for the three largest individuals. In April of 1967 only six *Corella* settled on the one-month plates, and the size given is the largest of those six. The mean size for November is abnormally small, probably because it represents less than a whole month's growth. The breeding stock of *Corella* on frame B was very small until those individuals which were still immature in October grew to reproductive size, sometime before November 20. Thus recruitment of the tadpoles to the plates throughout the month of November was unlikely; the 604 individuals on the one-month November plates probably represent settlement for only the prior two weeks or so.

In Figure 4 the size values for the first month's growth of *Corella* for each month of the year have been plotted against temperature. Since surface temperature readings were taken only once a month at Bremerton, it was thought that these readings might show enough variability to mask average seasonal trends. Therefore, along with the Bremerton temperature readings are plotted average bimonthly surface temperature readings for Point Jefferson (Megia, 1956). The Point Jefferson and Bremerton curves agree fairly well, although as might be expected, higher summer temperatures occur in the inshore waters at Bremerton. Figure 4 shows that there is a strong correlation between the initial month's post-larval growth and temperature. Seasonal food supply is very likely also an important factor here, but it was not measured in the present study. The correlation between morphological changes occurring after metamorphosis and the rate of growth of *Corella* is currently being investigated.

Predation on Corella

Eurylepta leoparda Freeman, a cotylean polyclad flatworm, was found inside a few adult *Corella* on the settling frames and floats at Bremerton during the September, November, December, March, and April visits. The worms were 1–3 cm. in length; laboratory observations (about 20–25) showed that they always entered the *Corella* by rolling into a tube as they passed through the branchial (incurrent) siphon (never the atrial siphon). Once inside the *Corella,* the flatworms unrolled themselves and usually ingested the branchial basket first; then they might either leave the individual through a siphon or continue devouring the remainder of the internal organs within 3–7 days, finally leaving only an empty test. Ingestion occurred by extrusion of the pharynx and sucking in of the food material. Generally only one flatworm was found inside a *Corella* at Bremerton, but occasionally there were two, and in the laboratory three or four often moved into the same *Corella.*

There are very few references concerning predation on ascidians. The polyclad flatworm *Cycloporus papillosus* feeds on the colonial tunicates *Botryllus* and *Botrylloides* (Jennings, 1957), and several species of molluscs have been observed to feed on simple and colonial ascidians (Thompson and Slinn, 1959; Miller, 1961; Barrett and Yonge, 1964; Ghiselin, 1964). Goodbody (1963) states that young *Ascidia nigra* are "undoubtedly" eaten by flatworms and by young polychaetes. To my knowledge, there is only one recorded observation of a flatworm feeding on adult simple ascidians. Crozier (1917) reported that *Pseudoceros crozieri* was found in the branchial sac of *Ascidia curvata* and *A. nigra* as well as on colonies of *Ectinascidia turbinata.* According to Crozier, each of the three sets of flatworms taken from the three species of ascidians would feed only on the particular species on which it had originally been found, that is, there were three "physiological varieties" of *Pseudoceros crozieri.* A few feeding experiments carried out with *Eurylepta leoparda* indicate that it will feed only on adult *Corella* and not on *Ascidia callosa, Chelyosoma productum,* or *Diplosoma macdonaldi,* three other species of ascidians presented as food. Conversely, other species of polyclads that have been collected have not been observed to feed on *Corella.* Interestingly, *E. leoparda* has been found only with the Bremerton population of *Corella.* In other local areas, such as Edmonds and Friday Harbor, this flatworm is apparently absent from the *Corella* populations, which show much more marked fluctuations in numbers than does the Bremerton population.

CONCLUSIONS

Corella's position in the successional sequence in fouling communities appears to be the following. It is apparently a primary colonizer, since the tadpoles have been shown to settle preferentially on clean, unfouled surfaces. It is a "fugitive" species; the tadpoles settle on new or denuded surfaces and grow quickly to sexual maturity. As they die off, however, colonial ascidians may take over the space, overgrowing small *Corella,* particularly during the winter, and thus preventing the maintenance of stable populations of *Corella.* Tadpoles tend to settle in the immediate vicinity of the adult *Corella,* however, thus prolonging the length of time that *Corella* can exist before being replaced.

The dominant organisms on the older floats (submerged for five years or more) are *Metridium senile, Eudistylia vancouveri, Mytilus edulis* and *Pyura haustor.* All of these species, with the exception of *M. senile,* settled sparsely on the frames; only at the termination of observations were they beginning to become more evident. Judging from observations of the older floats, had the frames been left submerged these species would probably have continued to increase in abundance. On the older floats there is, however, a seasonal sloughing off of some of these organisms. These spots are usually then colonized by *Corella.* The populations of *Corella* in shady locations are able to survive the spring period of diatom overgrowth better than other populations and may be partially responsible for the summer recolonization by *Corella* of the denuded areas.

SUMMARY

1. A one-year field study of the ecology of the solitary ascidian *Corella willmeriana* Herdman was conducted between April, 1966, and April, 1967, at the Bremerton Yacht Club, Bremerton, Washington, where two polyvinyl chloride frames containing glass plates were examined at monthly intervals.

2. The results indicate that *Corella* is a primary colonizer, preferring to settle on clean surfaces. Growth is rapid during the summer, when sexual maturity, corresponding to a size of 12 mm., may be attained in three months and life span is approximately five months. Individuals grow at a slower rate and live longer during the winter; the life span then is seven or eight months.

3. Very young specimens of *Corella* are frequently overgrown during the winter by the colonial ascidian *Diplosoma macdonaldi.* The causes of death of adult *Corella* are not completely known, although a small percentage of them are eaten by the polyclad flatworm *Eurylepta leoparda.* A luxuriant spring growth of filamentous diatoms may cause death of adult *Corella* by smothering them.

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FEEDING AND OXYGEN UPTAKE OF THE TROPICAL SEA URCHIN EUCIDARIS TRIBULOIDES (LAMARCK)[1, 2]

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Feeding and oxygen uptake of echinoids have been studied by a number of investigators (for a recent review see Boolootian, 1966), but little work has been done on cidaroid urchins. These urchins are particularly interesting since they belong to an ancestral group from which all echinoids evolved (Durham and Melville, 1957).

There are over 150 living species and varieties in the family Cidaridae (Mortensen, 1928). Most of these live in the bathy-benthic zone, but some live in the littoral zone and a few live at great depths. In the West Indian region there are 12 species of cidaroids, one of which is littoral, the rest being found between 30 and 2000 meters (Mortensen, 1928). The littoral species, *Eucidaris tribuloides* (Lamarck), is relatively abundant in some habitats off southeastern Florida, particularly on coral reefs and rocky areas. It is easily maintained in aquaria where it grows at rates at least comparable with, and sometimes greater than those in the field (McPherson, in press). It is thus well-suited for laboratory studies.

This study was made to determine some of the factors which are important in the metabolism of *Eucidaris tribuloides*. Such parameters as temperature, season, size, and nutrition and their effect on oxygen uptake have been considered. Food preference and rate of feeding have been measured under laboratory conditions. This work will form a basis for studies on metabolism of deep-water cidaroids.

METHODS AND MATERIALS

Urchins used in respiration and feeding experiments were collected on Margot Fish Shoal, a "patch" coral reef several miles east of Elliot Key, Florida. After they were brought in from the field they were allowed to acclimate to aquarium conditions for at least several days before being used in experiments.

The food preference of *Eucidaris tribuloides* was tested for the boring sponge *Cliona lampa,* turtle grass (*Thalassia testudinum*), several algae (including *Dictyota* sp., *Liagona* sp.), and fish strips (mullet, *Mugil cephalus*). Eight rocks were arranged in a circle within a clean holding tank. The rocks were placed so that each was separated from the next by about 10 to 15 cm. The diameter of the circle of rocks was approximately one meter. Four of these rocks were infested with the boring sponge *Cliona lampa* or were placed on the other foods to be tested. Alternating with these were four rocks of similar size, but without any evident algal or

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animal growth. Sea water flowed into the tank, circulated around the tank and flowed out at a drain in the center of the circle of rocks. Urchins were placed in the center of the circle and the following day their positions were recorded in relation to the food. Those touching a non-food rock (control) were recorded as R. Those not touching any rock or food were recorded as 0. This was repeated on several days for each food with fresh urchins. The position of each rock was moved one place each day in the circle.

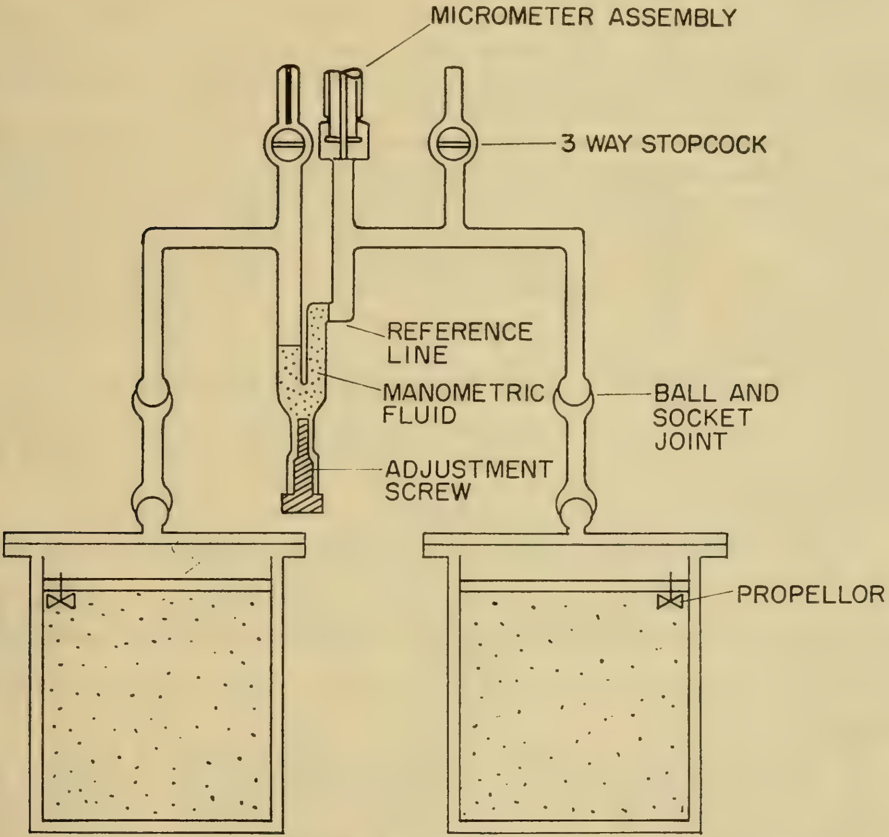

FIGURE 1. Schematic drawing of modified Scholander volumetric respirometer.

Before determining feeding rates urchins were maintained in aquaria two to three months and were offered an excess of rock infested with *Cliona lampa.* Feeding rate was determined in the laboratory on these individuals held in 950-cc. polyethylene cages. Weighed portions of rock infested with *Cliona lampa* were put into the cages and at the end of eight days the rock was reweighed (wet) and the amount eaten determined by differences between first and second weighings. Controls indicated that weight loss of rock during the experimental periods was not significant. Flow of sea water into aquaria was maintained in all feeding experiments at about two liters per minute.

Oxygen consumption was measured with a modified Scholander volumetric respirometer (Fig. 1). This consisted of a differential syringe manometer modified for attachment to two glass vessels (manometer obtained from RGI Inc., Vineland, New Jersey). The micrometer syringe had a total capacity of 200 microliters in 0.2 microliter divisions. The manometer was attached to the vessels by ground glass ball and socket joints. Vaseline was used as a lubricant for the joints and for the ground glass top and lip of the vessels. Hexanol with rodamine B dye was used as the manometer fluid.

The vessels contained a plastic ring with a magnetic stirrer. During respiration experiments small motors outside the vessels were used to stir and aerate the water. Water movement appeared to be an important factor for the well-being of urchins (Moore and McPherson, 1965).

The total volume of a vessel and its connecting tube was about 955 cc. The plastic ring had a volume of 120 cc. The air volume was maintained at about 65 cc., so that there was about 770 cc. in the vessel for sea water and for the urchin. The volumes of the urchins used ranged from about 5 to 80 cc., leaving 765 to 690 cc. of sea water, respectively. One vessel contained the experimental animal while the other (control vessel) contained only sea water. Carbon dioxide released by the urchin would be absorbed in the sea water, and would not increase significantly in the gas phase.

The general procedure for a respiration experiment was as follows:

(1) Sea water was put in both vessels and well aerated at a constant temperature (in a controlled-temperature bath which held the temperature to ±0.01° C.) for several hours.

(2) An urchin was placed in one vessel.

(3) The magnetic stirring apparatus was placed in both vessels and the water levels adjusted.

(4) The vessels were closed and connected to the manometer and then immersed into the controlled-temperature bath, so that the major air spaces were submerged.

(5) The stop cocks were left open so that both vessels were connected to the atmosphere.

(6) The sea water in the vessels was stirred with the magnetic stirrers.

(7) After about 60 minutes the vessels were closed to the atmosphere and readings were made every five or ten minutes for 30 minutes. In a few cases readings were made over periods longer than 30 minutes. The standard error for the mean oxygen uptake in cc. per hour per gm. dry weight, determined from 27 readings on six urchins at 20° C., was ±0.03.

(8) Determinations of oxygen uptake were made during the day, between the hours of 0900 and 1400.

After respiration and feeding rate experiments the test diameter of each urchin was measured. A few of these urchins were saved for later tests, but most were examined to determine sexual condition, gut content, and tissue weight. Sex and the presence or absence of ripe sex cells were determined by microscopic examinations of gonadal smears. Gonads of each individual were removed, measured volumetrically in a graduated cylinder and dried in an oven. Gonad index was calculated from the gonadal volume and test volume following the method of McPherson (in press). In order to determine the total dry tissue weight, the remaining parts of the urchin, with the exception of the coelomic fluid, were decalcified with 20% formic acid. An ion exchange resin (WIN 3000) was added to facilitate the removal of calcium from the tissue, following a method used for decalcification of teeth at the University of Miami Medical School. A test indicated that the dry weight of the coelomic fluid was negligible (less than 1% of the total weight of the somatic tissue). Tissue of the urchin was dried for 24 hours at 105° C.

The ash-free dry weight of both the urchin and its food, rock infested with *Cliona lampa,* was measured in several cases. The rock was weighed and decalcified by the same method described above for *Eucidaris tribuloides.* The decalcified and dried residue of either of these was burned in a muffle furnace at 500° C. for 24 hours, and then reweighed. The difference between the dry weight before and after burning was used as an estimate of the ash-free dry tissue.

RESULTS

1. *Food preference*

In a starved state *Eucidaris tribuloides* would consume practically anything including wooden and even fiber-glassed sides of holding tanks. Under normal feeding conditions, however, these urchins showed a preference for the sponge *Cliona lampa* (rock) over *Thalassia testudinum,* algal-covered rocks, and strips of fish (mullet) (Table I). Sixty-six per cent of those urchins tested were associated with the *C. lampa* as compared with 24% not associated with this food. In other tests 26% were associated with *T. testudinum* compared with 74% not associated with food; 26% were associated with algal covered rocks compared with 74% not associated with food; and 32% were associated with fish strips compared with 68% not associated with food. Because of this preference in the laboratory and because

TABLE I

Food preference of Eucidaris tribuloides in the laboratory with the foods: Cliona lampa rock (C), Thalassia testudinum (T), algal rock (A), and fish strips (F). R = clean rock; O = urchins not in contact with any of the above items. Chi square $(\chi)^2$ *used to test significance of differences between the number of urchins associated with a food (rock) and those associated with a clean rock (control)*

Total number tested	Percentages			x^2
	C	R	O	
169	66	15	19	32.4
	T	R	0	
145	26	28	47	0.1
	A	R	0	
57	26	18	56	1.4
	F	R	0	
65	32	43	25	1.1

hard material was an important constituent of the gut content of urchins from the field, rock infested with *Cliona lampa* was selected as the food to be used in feeding rate and oxygen uptake experiments.

2. *Feeding rate*

Figure 2 gives the weight in gm. of wet rock (*Cliona lampa*) ingested per day for well-fed urchins during the period 1 to 9 February 1966. Urchins feed primarily on the surface of rock infested with *Cliona lampa,* ingesting both rock and sponge. Since the sponge tissue is concentrated at the surface and decreases with depth into the rock (Neumann, 1966), the urchins obtain relatively large amounts of tissue for the rock consumed. When the ash-free dry weight of the urchin and the rock consumed were determined, the intake corresponded roughly to 5% of the ash-free dry weight of the urchin per day.

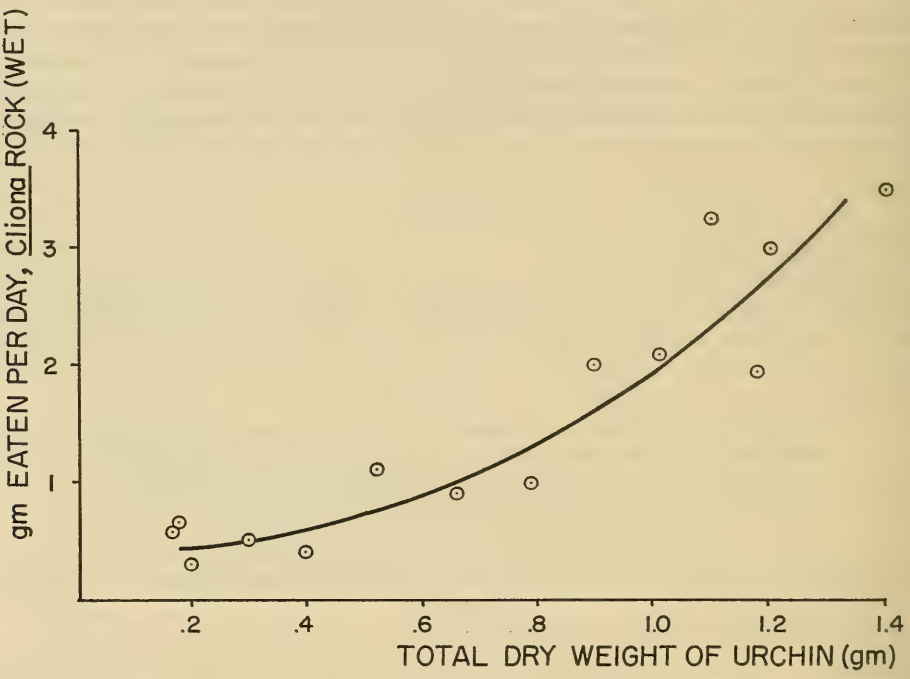

FIGURE 2. Feeding rate of well-fed individuals of *Eucidaris tribuloides* on rock infested with the boring sponge *Cliona lampa.* Experiment was made in aquaria during winter. Mean temperature 17° C. Line fitted by inspection.

3. *Preliminary experiments on oxygen uptake*

Tests were run to determine if changes in oxygen partial pressure or metabolic products would significantly change the rate of oxygen uptake of *Eucidaris tribuloides.* This was done by measuring oxygen consumption over periods of time considerably longer than normal respiration experiments. If changes in either of these factors significantly affected respiration during the normal tests then their effects should have been even more obvious in longer tests. The actual decrease in oxygen dissolved in the sea water that occurred during a test was measured in two cases. The micro-Winkler method described by Slack (1965) was used to measure the dissolved oxygen.

Urchins held in respirometers for varying periods of time over two hours showed some slight variation in oxygen consumption, but there appeared to be no significant trends in these tests. This was taken as an indication that any changes in the concentration of oxygen, carbon dioxide, metabolites or waste products in the respirometer water during these periods did not result in a measurable effect on the respiration of the urchin.

There was a 6% decrease in the dissolved oxygen in the respirometer water during an hour as indicated by micro-Winkler measurements. This small decrease in the dissolved oxygen in the respirometer water in spite of the oxygen consumed by the urchin is explained by the fact that there is a large source of oxygen in the respirometer vessel (compared with that used by the urchin). As the oxygen in the water is removed by the urchin it is replaced rapidly by oxygen in the air over the water due to the action of the magnetic stirrer. As stated, the volume of air over the water in the respirometer was about 65 cc. Since air contains about 200 cc. of oxygen per liter, each vessel before a run would contain about 13 cc. of oxygen in the air and about 5 cc. of oxygen dissolved in the water, depending on the temperature. If an average urchin removed 0.5 cc. of oxygen per hour, the loss at the end of two hours would be 1.0 cc. or only about 6% decrease in the total oxygen. This value is very close to the observed decreases.

TABLE II

O_2 consumption of Eucidaris tribuloides (well-fed and starved individuals). Urchins fed Cliona lampa rock. Temperature 30° C.

T.D. (mm.)	Well-fed	cc. O_2 per hour				
		Days of starvation				
		1	2	3	4	5
42	1.23	1.00	0.96		0.98	
26	0.46	0.34	0.34			
20	0.23	0.20				
28	0.65	0.65		0.28		
36	0.86	0.61		0.59		0.60
30	0.74	0.57	0.58			0.55

4. Oxygen uptake and nutrition

The effect of nutrition on oxygen consumption was studied by comparing the respiration of well-fed and starved urchins. Well-fed urchins were defined as those which were exposed to the food *Cliona lampa* and had this material in their gut. Starved urchins were defined as those which had been offered *Cliona lampa* in the laboratory, and which were then removed from this food for varying periods of time and held in clean polyethylene cages.

Respiration was measured individually on six well-fed urchins. These urchins were starved, and on one or more successive days their oxygen consumption was

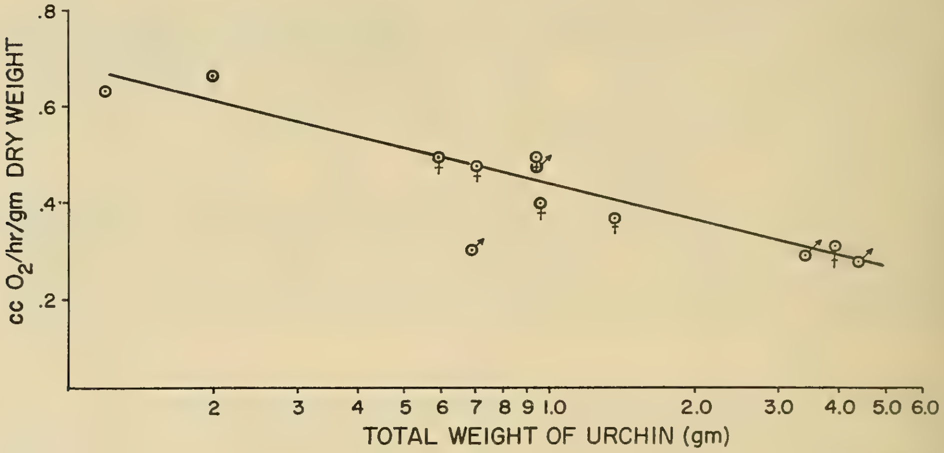

FIGURE 3. Relative oxygen uptake of *Eucidaris tribuloides*. Experiment was made in winter at 20° C.

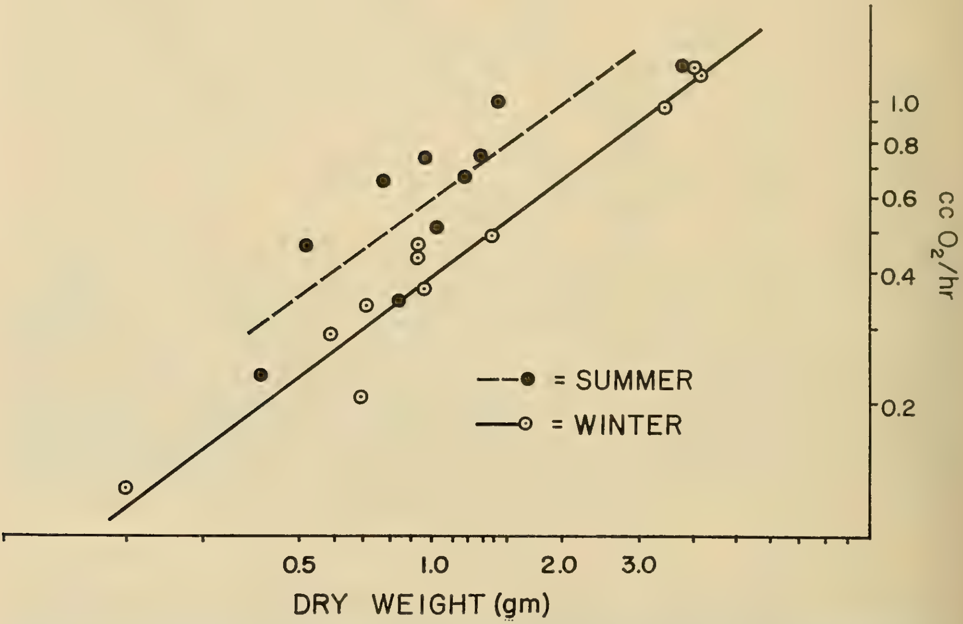

FIGURE 4. Oxygen uptake of *Eucidaris tribuloides* plotted against body size during summer at 30° C. (b = 0.65) and during winter at 20° C. (b = 0.70). Urchins well-fed.

remeasured. This test indicated that oxygen uptake decreased markedly during the first day of starvation, but showed little change over the next few days (Table II). The mean oxygen uptake decreased 22% from the original rate during the first day. Since oxygen uptake was relatively stable during at least the first one to five days of starvation, this interval of time was selected as a standard starvation period for further work.

5. *Oxygen uptake and reproduction*

Gonadal condition and sex were determined for most of the urchins in which oxygen consumption was measured. There was no indication that these factors significantly affected respiration. In addition there was no significant correlation between relative oxygen uptake and gonad index.

6. *Oxygen uptake and size*

Relative oxygen consumption plotted against body weight showed the expected trend of decreasing oxygen uptake with increasing body size (Fig. 3). The smallest urchins tested respired about twice as fast as did the largest urchins, the latter being about 30 times as large as the former.

The relationship between metabolic rate and body size may be expressed in the formula (Bertalanffy, 1957):

$M = a\ W^b$, where M = cc. O_2 per hour consumed by the urchin
W = weight of urchin
b = log-log slope of the line which relates O_2 uptake to weight
a = the point at which the above line intercepts the ordinate M.

Figure 4 gives the oxygen consumption of *Eucidaris tribuloides* plotted against size at 20° and 30° C. (winter and summer). The slopes of the resulting lines are 0.70 (winter, 20° C.) and 0.65 (summer, 30° C.). These values indicate an oxygen uptake which is almost proportional to surface area (or $\frac{2}{3}$ power of weight). In some echinoderms "b" has been shown to decrease with increasing temperature, indicating that larger animals consume proportionally less oxygen than smaller animals as the temperature increases (Farmanfarmaian, 1966). This may be related to decreased ambient oxygen or increased oxygen demand but insufficient supply.

7. *O_2 uptake and temperature*

Two aspects of the effect of temperature on respiration were considered. First, the short-term effect of temperature change on oxygen consumption was measured for some individuals in order to determine the time required for respiration to reach a relatively stable rate. Urchins were taken from water with an ambient temperature of 28–29° C. and placed in respirometers at temperatures of 10°, 15° 20°, and 30° C. The oxygen uptake of urchins placed in water which was cooler than ambient fluctuated during the first 40 minutes, and after this remained relatively stable (Fig. 5). Oxygen uptake was low during the first 10 minutes, then rose to a maximum in 20 to 30 minutes, and then decreased to an intermediate value after about 40 minutes. The oxygen uptake of urchins tested at temperature near ambient showed little change in respiratory rate over the same interval (Fig. 5). Second, the effect of temperature on the stabilized rate of oxygen consumption was measured at the following temperatures: 10°, 11°, 15°, 20°, 24°, 26°, and 30° C. Urchins were placed in the respirometers at these controlled temperatures and allowed to acclimate for one hour, after which oxygen determinations were made. The urchins used in these tests were within the size range 28 to 35 mm. After each test the dry weight of the urchin was determined and the respiration was expressed as cc. oxygen per hour per gm. dry weight (Figs. 6 and 7). The mean oxygen consumption for each temperature and the Q_{10} for each 5° C. interval between 10° and 30° C. were then calculated. Both starved and well-fed urchins were used (but these were considered separately).

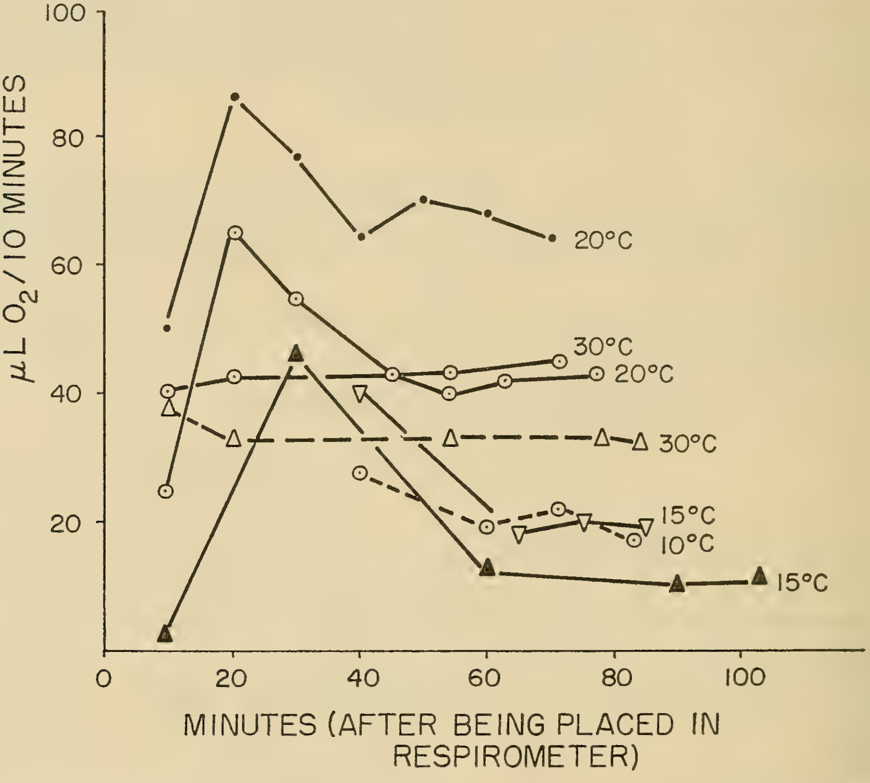

FIGURE 5. The immediate effect of temperature change on the oxygen uptake of *Eucidaris tribuloides*. Urchins were moved from ambient water with a temperature of 28–29° C. to water in the respirometer at different temperatures.

The stabilized rate of oxygen consumption of *Eucidaris tribuloides* at different temperatures was measured in both summer and winter to determine if there were seasonal acclimation. The log of the mean oxygen consumption for each of the temperatures given above was plotted following the method given by Prosser and Brown (1961), and the degree of translation and rotation of the resulting curves for winter and summer was described (Figs. 6 and 7).

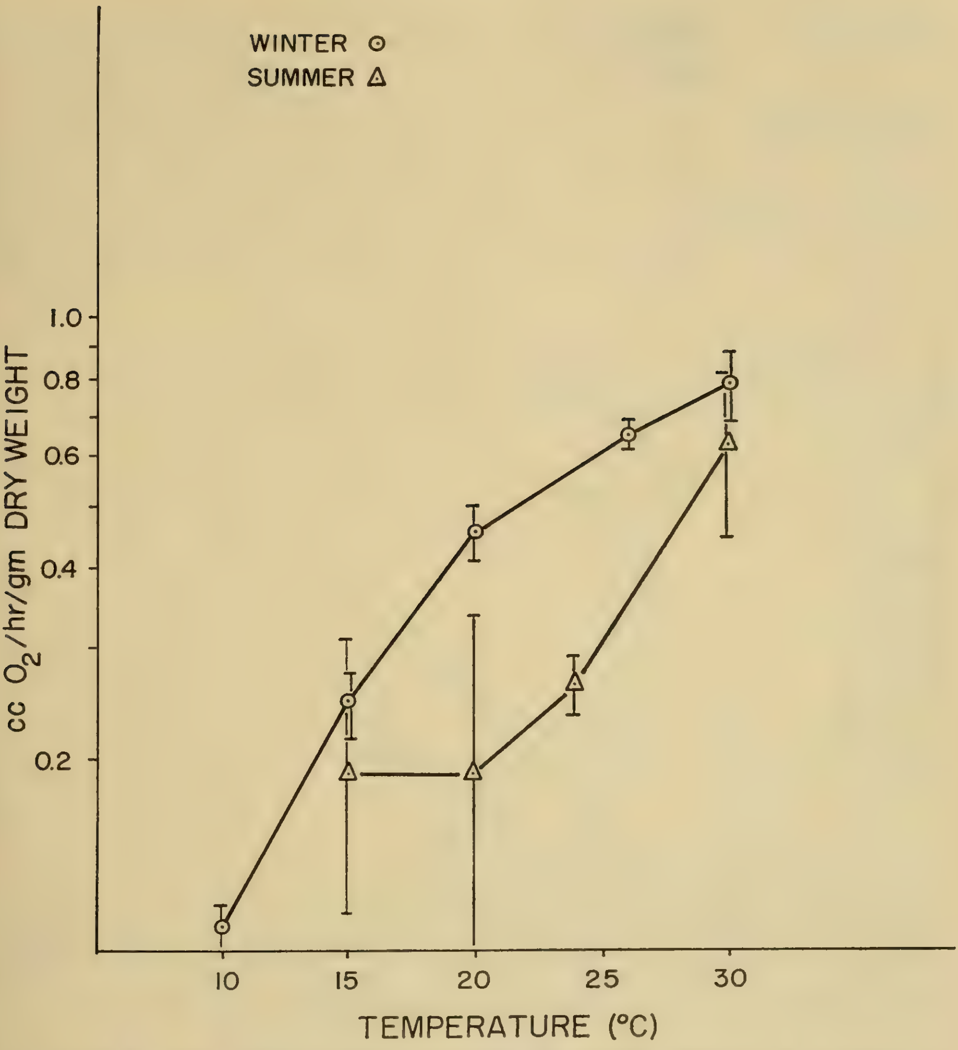

FIGURE 6. Mean oxygen uptake of individuals of *Eucidaris tribuloides* at stabilized rates for different temperatures. Urchins well-fed. The vertical lines give the 95% confidence intervals.

The Q_{10} of well-fed urchins increased rapidly in summer and decreased in winter as the temperature was increased from 15° to 30° C. The Q_{10} of starved urchins over a similar temperature range was more variable and manifested less overall change than that of the well fed individuals. In summer it increased 1.1 units in the temperature range 15° to 30° C. as compared with 3.4 units for the well-fed urchins. In winter it was essentially the same at high and low temperatures (Table III). These facts indicate that in both summer and winter well-fed

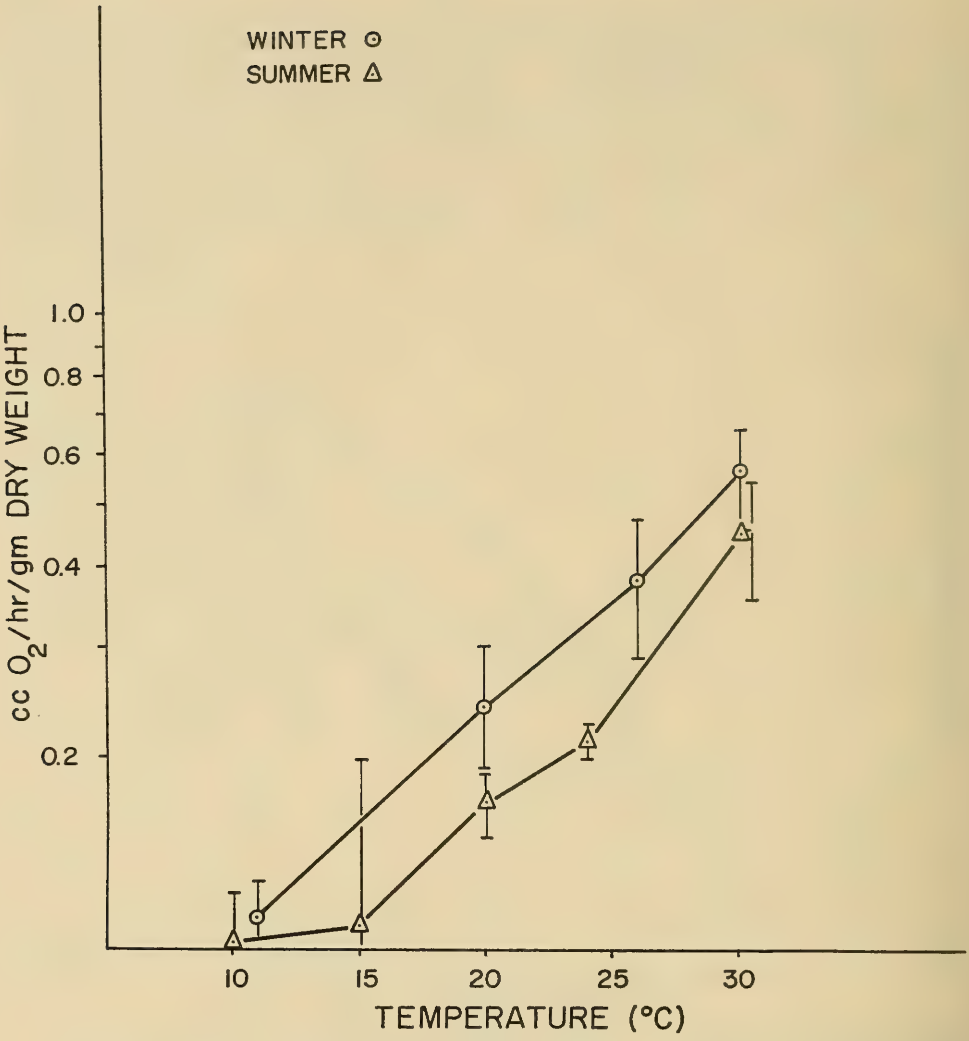

Figure 7. Mean oxygen uptake of individuals of *Eucidaris tribuloides* at stabilized rates for different temperatures. Urchins starved. The vertical lines give the 95% confidence intervals.

urchins were probably more sensitive to temperature changes in their stabilized respiratory rates than were starved urchins.

Both starved and well-fed urchins exhibited partial seasonal acclimation in which oxygen consumption curves were well above summer curves at the corresponding temperatures. The acclimation was not complete since the mean summer oxygen consumption was higher at the summer ambient than the mean winter oxygen consumption at the winter ambient. The difference between the mean summer and winter oxygen consumption curves of the starved urchins was less than that between the mean summer and winter curves of the well-fed urchins. This suggests that the process of feeding or digestion may play an important part in acclimation over normal environmental temperature ranges.

At 30° C., which is close to the summer ambient, the oxygen consumption curves tended to converge while at the winter ambient of 20° C. they were widely separated. This pattern resembled what Prosser and Brown (1961) have called translation and clockwise rotation (convergence at higher temperatures). At temperatures below the winter ambient there was a second convergence of oxygen consumption curves, so that around 10° C. there was little difference in the O_2 consumption of winter or summer urchins (no acclimation).

TABLE III

Q_{10} of well-fed and starved urchins (Eucidaris tribuloides) in winter and summer. Determined from changes in the mean oxygen uptake given in Figures 6 and 7

Well-fed		Starved	
Temperature, ° C.	Q_{10}	Temperature, ° C.	Q_{10}
Summer		Summer	
15–20	1.0	10–15	1.2
20–35	2.2	15–20	2.4
25–30	4.4	20–25	1.7
		25–30	3.5
Winter		Winter	
10–15	5.2	11–15	2.5
15–20	3.4	15–20	2.1
20–25	1.8	20–25	2.3
25–30	1.6	25–30	2.7

DISCUSSION

Eucidaris tribuloides feeds on hard particles of limestone rock deriving nourishment from associated organisms (McPherson, in press). Since boring and encrusting sponges are prevalent on rocks in tropical seas these might well be important in the nutrition of this urchin. The preference of *E. tribuloides* in aquaria for the boring sponge *Cliona lampa* might be an indication of the importance of sponge in the diet of this species.

The relatively high rate of feeding observed in the laboratory indicates that *Eucidaris tribuloides* is a potential erosive agent. Urchins apparently fed throughout the year as indicated by the presence of food in individuals collected monthly in the field, and by observations of feeding in aquaria throughout the year (McPherson, in press). Urchins, of the mean size of those found on coral reefs, consumed about one to two gm. of wet carbonate rock per day in laboratory experiments. The feeding rate in the field, however, is probably less than that measured in the laboratory, as indicated by a slower growth rate in the former area than in the latter (McPherson, in press). Lewis (1964) stated that carbonate ingestion by *Diadema antillarum* may be of some importance in the process of erosion of certain zones of the coral reef. In this habitat he determined that *D. antillarum* ingested a mean weight of at least two gm. of carbonate per day. This intake in an area of high density, such as that reported by Randall *et al.* (1964) of 13.4 urchins per square meter off the Virgin Islands, would result in the removal of a considerable amount of coral rock. Erosion caused by *E. tribuloides,* however, would probably be much less than that caused by *D. antillarum* since the density and feeding rate of the former are probably much lower than that of the latter. McPherson (in press) reported a maximum mean density for *E. tribuloides* of two per square meter on French Reef, Florida. In other places off southeastern Florida densities were much less than that observed on this reef.

Under the experimental conditions used in this study food, size of organism, and temperature were important factors influencing the oxygen uptake of *Eucidaris tribuloides.* This is not surprising since these factors are all generally important in the metabolism of invertebrates (Zeuthen, 1947). It is interesting, however, that *E. tribuloides* does manifest some control over its oxygen uptake when temperature changes. Sudden changes in temperature resulted in a rather unstable rate of oxygen consumption, but this only lasted some minutes, and in an hour the rate of respiration became relatively stable. Slow changes in temperature, such as those which occur seasonally, had only a slight effect on respiration since the urchins partially acclimated during this time. Partial control of respiration would allow these animals to maintain some independence from environmental temperature.

Farmanfarmaian and Giese (1963) found that *Strongylocentrotus purpuratus* from ambient water of 14–19° acclimated to water at 5° C. The oxygen consumption curves were similar to those described for seasonal acclimation of *Eucidaris tribuloides* with the cold-acclimated urchins having rates above the warmer living individuals (translation). The two oxygen consumption curves, for acclimated and non-acclimated urchins, also tended to converge at higher temperatures (clockwise rotation) as did those of *E. tribuloides.* Boolootian and Cantor (1965) reported that *Arbacia punctulata* acclimated to low temperatures did not have a higher O_2 consumption than urchins maintained at higher temperatures. They stated that this suggested that *Arbacia* and *Strongylocentrotus* have different physiological mechanisms for thermal acclimation.

There was little variation in oxygen uptake between individuals of *Eucidaris tribuloides* at temperatures well below their winter ambient (10° and 15° C.). Even urchins tested in different seasons and in different nutritional conditions had roughly the same oxygen consumption for that particular temperature used in the experiment. Processes involved in nutrition and acclimation presumably had ceased or decreased to such low levels that their effect on respiration was negligible.

SUMMARY

1. In the laboratory *Eucidaris tribuloides* showed a food preference for rock infested with the boring sponge *Cliona lampa* over turtle grass (*Thalassia testudinum*), several algae, and fish strips.

2. In the laboratory, urchins consumed wet rock infested with *Cliona lampa* at rates ranging from less than one to over three gm. per day depending on the size of the urchin.

3. Oxygen consumption of urchins was measured in the laboratory with a modified Scholander volumetric respirometer.

4. Oxygen uptake decreased significantly during the first day of starvation, but then remained relatively stable during several successive days of starvation.

5. There was not a significant correlation between gonad index or sex of the urchin and relative oxygen uptake.

6. Relative oxygen consumption decreased with increasing size of the urchin. Values for "b" indicated an oxygen uptake which was almost proportional to surface area at 20° and 30° C.

7. Oxygen uptake fluctuated during the first hour when urchins were placed in water several degrees colder than their ambient water. At the end of this hour, however, the rate of oxygen uptake had become relatively stable. Well-fed urchins manifested greater changes in their stabilized rates of oxygen uptake (Q_{10}) at different temperatures than did starved urchins.

8. Urchins manifested partial seasonal acclimation of oxygen uptake as indicated by the fact that winter oxygen consumption curves were well above summer curves at the corresponding temperatures. The acclimation was probably not complete since mean summer oxygen uptake was higher at the summer ambient than the mean winter oxygen uptake at the winter ambient.

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DYNAMICS OF ECDYSONE SECRETION AND ACTION IN THE FLESHFLY SARCOPHAGA PEREGRINA [1]

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Ecdysone, the growth and molting hormone of insects, provokes a developmental response when it accumulates to "threshold titer" at certain "critical periods." Meanwhile, the titer of juvenile hormone dictates whether the developmental response will be molting or metamorphosis. These two generalizations epitomize the prevailing theory of ecdysone's action.

In support of the theory there is a wealth of evidence that the secretion of ecdysone is necessary for molting or metamorphosis. But whether the developmental response is triggered by a certain threshold titer of ecdysone is by no means assured. There remains the possibility that ecdysone is promptly and progressively utilized and inactivated after its release into the blood. Under that circumstance, the effects of the hormone might be cumulative within the target-organs without any marked change in its concentration in the blood or the insect as a whole.

Precisely this state-of-affairs appears to be the case when ecdysone provokes puparium formation of mature larvae of the fleshfly, *Sarcophaga peregrina.* In the present report we document the swift inactivation of ecdysone after its secretion into the blood. Moreover, we show that the overt developmental response, puparium formation, is triggered, not by the accumulation of ecdysone itself, but, rather, by a summation of the latent, covert effects of the hormone.

MATERIALS AND METHODS

1. *Experimental animals*

All experiments were carried out on larvae of *Sarcophaga peregrina.* This species was chosen for study because of its large size and the ease with which one can obtain unlimited numbers of carefully timed larvae. Thus, as documented by Ohtaki (1966) and diagrammed in Figure 1, the activation of the endocrine system is opposed or prevented as long as mature larvae are stored in contact with water. This inhibition is relieved when "wet larvae" are transferred to dry conditions. Therefore, the secretion of ecdysone can be timed from the "0-hour of dryness" until the initiation of puparium formation which, at 25° C, routinely takes place 16–17 hours later. Meanwhile, at any time during this period one

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can obtain homogeneous groups of larvae by timing their exposure to dry conditions.

All the larvae used in the present study were reared in gallon glass jars containing pig liver. After three days of feeding at 24.5 ± 1.5° C, the mature larvae emptied their crops and tried to crawl away from the food. For two further days they were stored in the same jars, to which water was added to maintain wet conditions. Then they were collected, washed in tap water, and placed in clean gallon jars containing sufficient water to cover the bottom. After two further days of storage at 24.5° C, the wet larvae were blotted with filter paper and either used immediately as "0-hour larvae" or aged in dry jars for two or more hours.

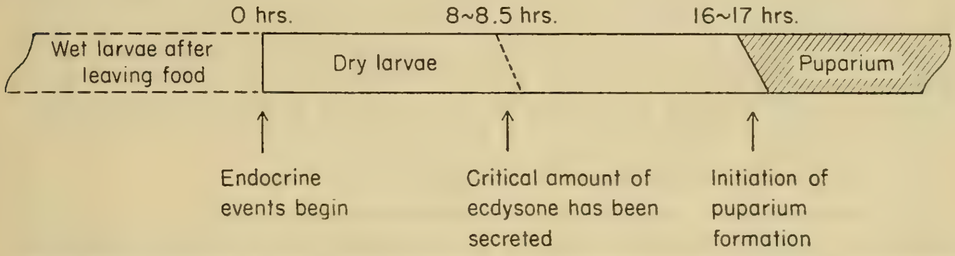
0 hrs.
8~8.5 hrs.
16~17 hrs.
Wet larvae after leaving food
Dry larvae
Puparium
Endocrine events begin
Critical amount of ecdysone has been secreted
Initiation of puparium formation

FIGURE 1. The timing of developmental events when mature larvae of *Sarcophaga peregrina* are transferred from wet to dry conditions at 25° C.

2. *The Sarcophaga assay*

After exposure to dry conditions for 0, 2, 4, or 6 hours, homogeneous groups of larvae were ligated just behind the sixth segment. The majority of assays were performed on "standard test abdomens" which were prepared as follows:

Larvae were ligated after exposure to dry conditions for six hours. Twenty-four hours later, individuals showing puparium formation in front of, but not behind, the ligature were collected and aged an additional 16 hours under dry conditions. Larvae showing any trace of puparium formation behind the ligature were discarded; the number that did so ranged from 1 to 10%. The others were immediately used as "standard test abdomens."

Each assay was performed on a homogeneous group of 10 to 20 abdomens. The body anterior to the ligature was cut away and 10 μl of the test solution were injected by means of a 30-gauge glass or stainless steel needle sealed to a 100-μl microsyringe; the latter was controlled by an Agla micrometer. The needle was inserted through a loose ligature which was tightened immediately after the injection. When tests were performed on entire larvae, the injection was made through a ligature placed across the anterior tip just behind the mouthparts.

3. *The "puparium index"*

All assays were controlled 24 hours after injection. As recommended by Karlson (1956), each abdomen was scored as having undergone complete, marked, slight, or no puparium formation. These reactions were equated to 100, 75, 50, and 0%, respectively, for the purpose of calculating the "puparium index."

4. *Ecdysone solutions*

Synthetic α-ecdysone was obtained from Drs. John Fried and John Siddall of the Syntex Research Laboratory. The crystalline hormone was weighed, dissolved in 1 part warm isopropanol, and diluted with 9 parts water. Twofold serial dilutions were prepared in 10% isopropanol. One series of experiments made use of the phytoecdysones, β-ecdysone and ponasterone A, obtained through the courtesy of Profs. K. Nakanishi and T. Takemoto, of Tohoku University.

5. *Collection and bioassay of larval blood*

By means of iridectomy scissors a small sliver of integument was cut from the dorsal tips of the abdomens of a homogeneous group of ten washed larvae, care being taken not to damage the gut or other viscera. The larvae were immediately placed in a tilted Petri dish into which the blood was expressed by the spontaneous contractions of the cut larvae. The blood was immediately drawn into a 100-μl syringe and 10 μl injected into each of a series of test abdomens.

6. *Extraction and concentration of ecdysone from the blood*

The method was a modification of that described by Kaplanis *et al* (1966). Blood was collected from ten larvae, as just described. The volume was recorded and the blood immediately ejected into a conical centrifuge tube containing 4 ml of a 1:1 mixture of acetone and absolute ethanol. In this same manner additional blood was collected until a total of 1.5 ml had been obtained from a homogeneous group of approximately 100 individuals. The mixture was stirred at room temperature and then centrifuged at 1000 *g* for five minutes. The supernatant was decanted and the precipitate extracted twice more with 2-ml volumes of the solvent mixture. The supernatants were pooled and filtered with suction through a sintered medium-pore filter into a 50-ml round-bottom flask. The filter was rinsed with the solvent mixture and the extract reduced to dryness *in vacuo* on a rotary evaporator with temperatures rising to 50° C.

Crude extracts of this sort are extremely toxic when injected into larvae. Therefore, further purification was necessary. To this end, the contents of the flask were rinsed with 70% methanol into a small separatory funnel and washed with an equal volume of petroleum ether (*b.p.* 37–60° C). The methanolic hypophase was drained into a small round-bottom flask and reduced to dryness at 50° C as just described. By means of *n*-butanol the contents were rinsed into a separatory funnel and an equal volume of aqueous 2% sodium carbonate was added. The mixture was stirred, centrifuged, and the butanolic hypophase collected. The aqueous epiphase was twice re-extracted with 0.5 volume of butanol. The alcoholic phases were combined and washed twice with 0.5 volume of water. The aqueous phases were collected and re-extracted with 0.25 volume butanol and the latter washed with 0.5 volume of water. The butanolic phases were once again combined, placed in a round-bottom flask, and reduced to dryness on a rotary evaporator with temperatures rising to 65° C. With small volumes of methanol the contents of the flask were rinsed into a vial and the solvent evaporated in a stream of nitrogen. The extracted material was redissolved in 0.15 ml water, corresponding to one-tenth the original blood volume. This tenfold concentrate was assayed by the injection of 10 μl into each of a series of isolated abdomens.

By the addition of known amounts of α-ecdysone the combined efficiencies of the extraction and purification procedures were found to be 90%. Therefore, in the present report, all extractions (except where otherwise noted) have been corrected for 10% loss.

7. *Extraction and concentration of ecdysone from the entire animal*

One to four larvae were homogenized in Ringer's solution and extracted with 4 ml of a 1:1 mixture of acetone and absolute ethanol. The mixture was centrifuged and the precipitate washed twice with a total of 4 ml of the solvent mixture. The supernatants were combined and the ecdysone extracted as described above for blood.

To harvest any ecdysone that might be complexed to proteins, the precipitate was reduced to dryness and treated with "Pronase." The reaction mixture consisted of 1 ml of 0.04 *M* Tris buffer (pH 7.5), 0.0066 *M* calcium chloride, and 1 ml of a 0.15% aqueous solution of the enzyme. After incubation at 40° C for 24 hours, the mixture was centrifuged and the supernatant decanted. The precipitate was twice washed with acetone-ethanol and the supernatants combined. The latter was purified and assayed as described above for blood. Additional extractions were also carried out on anterior and posterior ends of ligated larvae.

RESULTS

1. *The effects of massive injections of ecdysone*

As illustrated in Figure 1, 50% of larval abdomens undergo puparium formation when isolated after 8.5 hours of dryness. However, they require an additional 8 hours to do so. The significance of this latent period was examined in groups of 0-hour larvae injected with α-ecdysone, β-ecdysone, or ponasterone A. As summarized in Table I, even the largest doses failed to reduce the latent period to less than 8.5 hours.

2. *Ecdysone secretion during exposure to dry conditions*

Graded doses of α-ecdysone were injected into abdomens ligated after 0, 2, 4, and 6 hours of exposure to dry conditions. Each dose was tested in a homogeneous group of 20 abdomens. As shown in Figure 2A, the longer the preliminary exposure to dryness, the more sensitive were the abdomens to the injected ecdysone.

In Figure 2B the critical doses provoking a puparium index of 50% have been plotted as a function of the hours of exposure to dry conditions prior to ligation. These values define a *Sarcophaga* unit for abdomens isolated after 0, 2, 4, and 6 hours, respectively. Sensitivity to injected ecdysone increases at a rate that may be equated to about 0.007 μg per hour. It will be recalled that when ligation is postponed to the 8–8.5 hour, 50% of abdomens undergo puparium formation without any injection. Taken as a whole, these findings suggest that, at the outset of exposure to dry conditions, the endocrine system is activated and the ring-glands initiate the secretion of ecdysone at a fairly steady rate of about 0.01 μg per hour.

On the basis of Figures 2A and 2B one might suppose that abdomens isolated after 6 hours of dryness would be optimal for the biological assay. Unfortunately, this is not the case because up to 10% of the "6-hour abdomens" give false positive assays—*i.e.*, they undergo puparium formation without any injection.

TABLE I

Acceleration of puparium formation by injection of ecdysones into 0-hour larvae

Substance injected	Amount (μg)	Number injected	Time for 50% to initiate puparium formation (hrs)
Controls (solvent only)			
H_2O		20	17
10% Isopropanol		20	21
α-Ecdysone*	0.5	10	8.5
	1	10	8.5
	2	10	8.5
β-Ecdysone*	0.25	10	8.8
	0.5	10	9
	1	10	9
	2	10	9
Ponasterone A**	0.5	10	8.5
	0.7	10	8.5

* In 5 μl H_2O.
** In 5 μl 10% isopropanol.

Figure 2A also records the sensitivity of "standard test abdomens" which, as described above, are prepared by the further ageing of abdomens ligated after 6 hours of dryness. It will be observed that 95% of these abdomens have become less sensitive than at the time of their isolation. However, about 5% retain great sensitivity without showing any false positive assays. A puparium index of 50% is provoked by the injection of 0.035 μg α-ecdysone, which we define as a "*Sarcophaga* unit" for standard test abdomens (Ohtaki *et al,* 1967). This is the same average value that characterizes abdomens isolated and immediately used after 2 hours of dryness.

3. *Ecdysone assays of the blood*

On the basis of the foregoing results as well as Fraenkel's (1935) classic study of *Calliphora,* we anticipated that detectable amounts of ecdysone would be demonstrable in the blood. This prospect was tested by injecting standard test abdomens with blood obtained from donors at four successive stages—namely, larvae after 0, 6, and 12 hours of dryness; and 16-hour larvae showing the initiation of puparium formation. Each standard test abdomen received 10 μl of donor blood.

All assays were uniformly negative. So, by reference to Figure 2A, it appears that at no stage in puparium formation is as much as 0.0013 μg α-ecdysone present in 10 μl of blood. And since a mature larva contains 35 μl blood, the total ecdysone in the entire blood could scarcely exceed about 0.005 μg, *i.e.*, 15% of a *Sarcophaga* unit for standard test abdomens.

4. *Extraction of blood ecdysone*

In order to bring this low titer within the range of the biological assay, we undertook the extraction and partial purification of blood ecdysone as described under Methods. The extract was redissolved in distilled water as a tenfold concentrate of the original blood, and 10 μl were injected into each of a series of standard test abdomens.

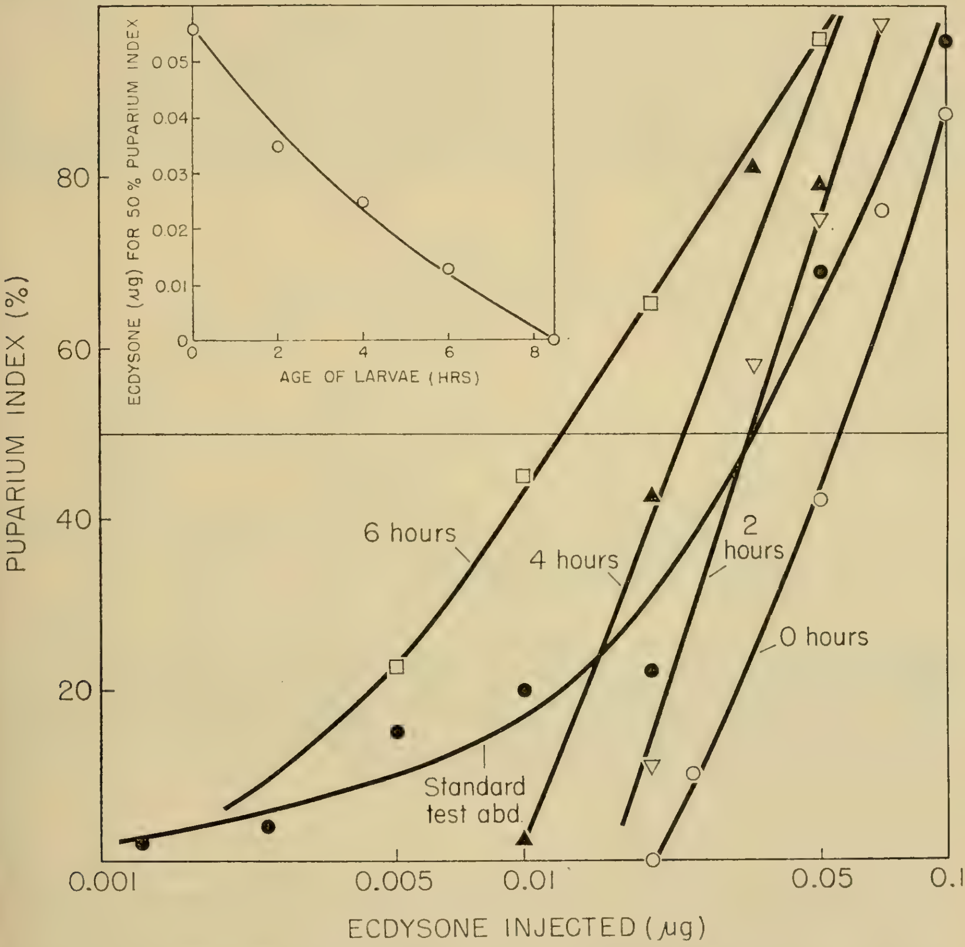

FIGURE 2A. The developmental responses (computed in terms of the puparium index) are plotted as a function of the amount of injected α-ecdysone. The curves correspond to abdomens ligatured after the indicated hours of exposure to dry conditions. For description of "standard test abdomens," see text.

FIGURE 2B. The inset in the upper left corner records the amount of injected ecdysone required for a 50% response of each type of abdomen.

Three concentrates of the blood of 0-hour larvae were assayed on a total of 41 standard test abdomens with the following result: 2 abdomens showed a trace of puparium formation; the other 39 were negative. This result corresponds to a puparium index of about 2%—a value which, as indicated in Figure 2A, is at the extreme limit of the calibration curve for standard test abdomens. Consequently, it appears that about 0.001 μg ecdysone is present in 10 μl of blood concentrate. On the assumption of 90% efficiency of the extraction procedure, we estimate that a total of 0.0004 μg ecdysone is present in the 35 μl of blood of a 0-hour larva, corresponding to about 1% of a *Sarcophaga* unit for standard test abdomens (see Table II).

The experiment was repeated on concentrates prepared from the blood of larvae at the 8th hour of exposure to dry conditions; 27 of 37 test abdomens showed no detectable reaction; 10 showed traces of puparium formation. This corresponds to a puparium index of 13% which, by reference to Figure 2A and on the assumption of 90% recovery, indicates the presence of about 0.0016 μg ecdysone in the entire blood of an 8-hour larva; *i.e.*, about 5% of a *Sarcophaga* unit (Table II).

Table II

Extraction of ecdysone from Sarcophaga peregrina

Stage	Ecdysone in:	
	Entire blood (μg)	Entire animal (μg)
0-hour larva	0.0004 (1%)*	0.0013 (4%)
8-hour larva	0.0016 (5%)	0.0025 (7%)
17-hour (white puparium)	0.005 (15%)	0.02 (57%)

* The bracketed figures record the corresponding percentage of a *Sarcophaga* unit as ascertained in assays performed on standard test abdomens.

In like manner the blood was collected from 100 individuals which had formed white puparia after 17 hours of exposure to dry conditions. Assays of the concentrated extract gave a puparium index of 34%, corresponding to about 0.005 μg α-ecdysone (15% of a *Sarcophaga* unit). This value is in accord with the upper limit of blood ecdysone calculated above in Section 3.

5. *Ecdysone in the entire larva*

The presence of so little ecdysone in the blood suggested that the hormone might be bound to the tissues. To test this possibility entire larvae were homogenized and extracted. The results summarized in Table II show that only about 4% of a *Sarcophaga* unit is present in an entire 0-hour larva, including the ring-gland itself. Even more surprising is the finding that only about 7% of a unit is present in an 8-hour larva, *i.e.*, at a stage where 50% of individuals have received all the ecdysone they require for puparium formation. The highest titer is found in white puparia where each individual contains about 57% of a *Sarcophaga* unit.

6. *Recovery of injected ecdysone from the blood*

Known amounts of α-ecdysone were injected into 0-hour larvae. Thereafter, at regular intervals, groups of injected larvae were sacrificed and their blood collected and assayed by injecting 5 μl into standard test abdomens. The puparium index was calculated in each case and equated to absolute units of α-ecdysone by reference to Figure 2A. The percentage of the injected ecdysone remaining in the blood was then calculated for each group.

The results summarized in Table III demonstrate that more than half of the injected hormone disappears from the blood within the first hour and that only traces remain after 8 hours.

TABLE III

Biological assays of blood obtained from larvae injected with α-ecdysone*

Ecdysone injected into blood donors (μg)	Time between injection and blood collection (hrs)	Number of test abdomens	Puparium index (%)	Equivalent ecdysone (μg)	% remaining in blood
2	1	10	100	>0.1	>35
	3	10	100	>0.1	>35
	5	20	62	0.047	16
	8	20	10	0.006	2
1	1	20	69	0.05	35
	3	20	23	0.015	11
	8	20	0	<0.001	<1
0.5	1	20	16	0.01	14
	3	20	8	0.004	6
	8	20	2	0.001	1

* Each standard test abdomen received 5 μl blood from donor larvae.

7. *Recovery of injected ecdysone from entire larvae*

One μg of α-ecdysone was injected into a series of 0-hour larvae. After exposure to room temperature for 0.1, 1, 3, or 8 hours, the entire larvae were homogenized and their ecdysone extracted and assayed in the usual way. Ninety per cent of the injected ecdysone was recovered from larvae extracted immediately after injection thereby testifying to the efficiency of the extraction and purification procedure. After correcting for this 10% loss, we found that one hour after the injection, only 50% was recovered; after three hours, 23%; and after eight hours, 2%.

The experiment was repeated except in this case the injected larvae were separated into blood and carcass. The blood was assayed by injecting 5 μl into standard test abdomens. The carcasses were cut open, rinsed in several changes of Ringer's solution, and then extracted and assayed in the usual manner.

The results summarized in Table IV once again document the rapid disappearance of injected ecdysone. And, here again, as in Table III, we note that the lower the dose, the more rapid is the relative rate of disappearance.

We entertained the possibility that ecdysone might be progressively bound to cellular proteins and in this manner rendered insoluble in the acetone-ethanol extraction mixture. To examine this possibility, 1 μg α-ecdysone was injected into each of ten 0-hour larvae. After three hours at room temperature, pairs of larvae were homogenized and five extracts prepared in acetone-ethanol. The precipitates were suspended in buffer and treated with "Pronase" as described under Methods. After incubation at 40° C for 24 hours, the reaction mixtures were once again extracted with acetone-ethanol to harvest any ecdysone that had been bound to proteins. These latter extracts were purified and assayed in the usual manner in a total of 54 standard test abdomens. All tests were uniformly negative.

Table IV

Recovery of injected α-ecdysone from blood and carcass of 1- to 8-hour larvae

Ecdysone injected (μg)	Time from injection (hr)	Number of animals	% of injected ecdysone recovered in		% not recovered
			Blood	Carcass*	
1.0	1	1	35	16	49
	3	2	11	13	76
	8	4	<1	<1	ca 99
0.5	1	1	14	9	77
	3	3	<3	<3	ca 95
	8	4	<1	<1	ca 99

* Corrected for 10% loss in the extraction procedure. Each experiment was performed in triplicate on the indicated number of animals. The average results are cited.

In control experiments, Pronase was found to have no deleterious effects on ecdysone. For example, two homogenates were prepared from uninjected larvae and 1 μg of α-ecdysone was added to each homogenate just prior to Pronase incubation. After 24 hours at 40° C, the reaction mixture was extracted with acetone-ethanol and purified in the usual way. The biological assays indicated the recovery of 100% of the added ecdysone.

For these several reasons the swift disappearance of injected ecdysone is accountable only in terms of its equally swift inactivation within the living insect.

8. *Stability of injected ecdysone at low temperature*

Three 0-hour larvae were placed at 2° C for 24 hours and then injected with 1 μg α-ecdysone. After an additional 24 hours at the low temperature, the larvae were homogenized and extracted. Biological assays of the extract showed the recovery of 100% of the injected hormone. Clearly, the inactivation of ecdysone is blocked by low temperature.

9. *Stability of injected ecdysone in the absence of oxygen*

Nine 0-hour larvae were placed in a covered Buchner funnel and exposed to a stream of nitrogen for 1 hour. Each individual was then injected with 1 μg α-ecdysone. After three additional hours in nitrogen, the entire larvae were homogenized and extracted. Assays showed the recovery of 90% of the injected ecdysone. Control experiments performed on entire larvae exposed to air showed the recovery of only 20% of the injected ecdysone. In additional control experiments, 0-hour larvae underwent full and complete recovery after 4 hours of exposure to nitrogen.

10. *Inactivation of ecdysone by larval fragments*

The gut was removed from four 0-hour larvae. These larvae were then cut into small fragments and placed in 2 ml Ringer's solution containing 1 mg streptomycin sulfate. The mixture was subdivided between two small flasks, one of which contained 3 mg of the potent anti-tyrosinase, phenylthiourea (PTU). One μg of α-ecdysone was added to each flask and the latter incubated at 25° C on a gyratory table. After 24 hours the ecdysone was extracted and assayed. The recovery was 40% in the mixture containing PTU and 22% in the mixture not containing PTU.

The experiment was repeated using a shorter incubation time and cut-up larvae from which the gut was not removed. After 8 hours, 94% of the added ecdysone was recovered from the mixture containing PTU and 70% from the mixture not containing PTU.

11. *Experiments on homogenates and subcellular fractions*

When the just-mentioned experiment was repeated using crude homogenates prepared from 0-hour larvae, 100% of the added ecdysone was recovered from all the reaction mixtures after 24 hours of incubation. The same was true when ecdysone was incubated with blood collected from 0-hour larvae.

On the assumption that ecdysone inactivation might be blocked by the presence of one or more endogenous inhibitors, the crude homogenate was fractionated prior to testing. To this end, two 0-hour larvae were cut into fragments and rinsed for 2 minutes in 2 ml 0.01 *M* HEK buffer (pH 7.4) made up in 20% sucrose (Kafatos, 1968, page 1258). The fragments were collected and homogenized in 2 ml of sucrose-free HEK buffer. The homogenate was centrifuged at 1000 *g* for 3 minutes. The supernatant was collected and centrifuged for 50 minutes at 200,000 *g*, and the particulate fraction was combined with the low-speed sediment. The insoluble fraction was diluted with 2 ml Ringer's solution. One mg streptomycin and 1 μg α-ecdysone were added to each reaction mixture and the latter incubated at 25° C on a gyratory table. Eight hours later, the ecdysone was extracted and assayed. Recovery of added hormone was 100% for both reaction mixtures. The experiment was twice repeated with the same result. Thus, as in the case of crude homogenates, ecdysone inactivation was not brought about by any of the subcellular fractions. This same negative result was observed in additional experiments in which 1 mg/ml of NAD or NADH was added to the reaction mixture prior to incubation.

DISCUSSION

1. *Ecdysone titer in relation to puparium formation*

At the outset of exposure to dry conditions a 0-hour larva contains 1.3 nanograms of ecdysone, most of which may be sequestered in its ring-gland (Table II). Fifty per cent of abdomens isolated at this time undergo puparium formation when injected with 55 nanograms of ecdysone (Fig. 2). Since the abdomen makes up 55% of larval mass, we calculate that an entire larva deprived of its ring-gland would require the injection of approximately 100 nanograms. When the ligation is postponed until the 8th hour of dryness, the ring-gland has already secreted sufficient ecdysone to cause 50% of abdomens to form puparia without any injection. Despite this fact, the amount of ecdysone in an 8-hour larva is only 2.5 nanograms (Table II). No less than 97 nanograms of ecdysone seem to have disappeared.

This same paradox is seen in the extractions and assays which Shaaya and Karlson (1965) and Shaaya (1967) have carried out on entire *Calliphora* larvae. From their data we calculate that at no stage prior to purparium formation does the titer of ecdysone exceed 26% of a *Calliphora* unit.

2. *The half-life of injected ecdysone*

All our experiments direct attention to the lability of α-ecdysone after its injection or its secretion by the ring-gland. Thus, as we have seen, within 1 hour after the injection of 1 μg, 50% had been inactivated; and after 8 hours, about 98% had been inactivated. The rate of inactivation was even higher in larvae injected with 0.5 μg. Therefore, for mature larvae of *Sarcophaga peregrina* at 25° C, we can state that the time for half-inactivation of α-ecdysone is less than 1 hour.

3. *The inactivating mechanism*

As described in Section 11 of Results, no trace of inactivation could be detected when α-ecdysone was incubated for 24 hours with larval blood or with crude homogenates prepared from 0-hour larvae. The same negative results were observed in tests performed on the soluble and the particulate fractions. By contrast, clear-cut inactivation occurred when α-ecdysone was incubated with larval fragments; *i.e.,* under conditions where the integrity of the cells was largely preserved. This demonstrates that the inactivating mechanism is present in at least some and perhaps most cells. As mentioned in Section 10 of Results, inactivation was brought about by larval fragments, irrespective of whether the gut was present or absent. Moreover, in additional experiments not mentioned above, inactivation of injected ecdysone took place in both anterior and posterior parts of ligated larvae. Inactivation by the anterior end was substantially faster than by the posterior end.

Our inability to demonstrate the inactivating mechanism in crude or purified homogenates implies the lability of the system or the requirement of co-factors and incubation conditions that were not satisfied. In Sections 8 and 9 of Results we observed that inactivation is blocked in larvae exposed to low temperature or to anaerobic conditions. Therefore, chemical and, more particularly, oxidative processes appear to be principally concerned.

4. *The dynamics of ecdysone action*

The rapid inactivation of ecdysone, demonstrated for the first time in the present study, directs attention to the accumulation, not of the hormone itself, but of the covert effects of the hormone. This new interpretation is summarized in the flow-sheet diagrammed in Figure 3. Ecdysone is secreted into the blood, taken up by the tissues, and reacts with the hormonal receptors to exert its primary action, whatever that may be. As a result of this primary reaction, a concatenation of biochemical and biophysical events is set in motion which comprise the covert effects of the hormone. The latter undergo, as it were, spatial and temporal summation within the target organs and finally trigger the overt effects, *i.e.*, the initiation of molting or metamorphosis. Meanwhile it may be noted that the covert effects are subject to decay if their temporal accumulation is interrupted short of the discharge level. This fact is documented in Figure 2A in terms of the lessened sensitivity to ecdysone injection of standard-test abdomens as compared to 6-hour abdomens from which they were derived. Moreover, the accumulation and implementation of the covert effects require a certain finite time. Thus, as shown in Table I, even the largest doses of α-ecdysone, β-ecdysone, or ponasterone A required at least 8.5 hours to provoke the initiation of puparium formation. This minimal "latent period" apparently corresponds to the time required for the covert effects of ecdysone to accumulate to the discharge level.

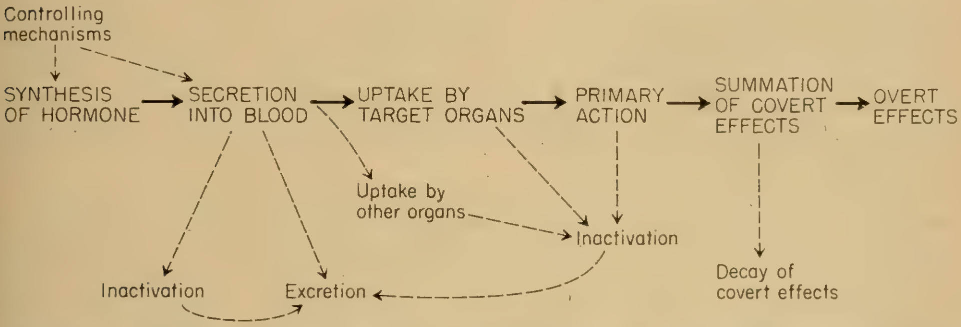

FIGURE 3. A revised theory of the hormonal action emphasizing the accumulation of the covert effects of ecdysone.

It has not escaped our attention that this conception of the dynamics of ecdysone action may be applicable to hormones in general.

5. *Transfusion versus parabiosis*

The literature pertaining to insect endocrinology is replete with carefully controlled experiments in which hormonal effects have been conveyed between donors and recipients by joining them in parabiosis. By contrast, there are few claims to accomplishing this same goal by the injection or transfusion of blood. One of these rare claims ranks among the most famous experiments in the history of insect physiology; namely, Fraenkel's (1935) induction of puparium formation by the injection of "pupation blood."

During the past 33 years the literature is silent in confirming or refuting this historic experiment. Now, for reasons set forth in the present study, it appears that Fraenkel's results were "false positives" such as were described above in Section 2 of Results. This seems all the more likely since Fraenkel's experiments were not controlled by the injection of any materials other than "pupation blood." Needless to say, our very ability to resolve this paradox owes a great deal to Fraenkel's insightful pioneering work.

We gratefully acknowledge the critical reading of the manuscript by Professor Lynn M. Riddiford.

SUMMARY

1. Ecdysone is in a highly dynamic state after its injection or its secretion by the ring-gland of *Sarcophaga peregrina*. Hormonal activity is rapidly destroyed by an inactivating mechanism which is present in the tissues but not in the blood.

2. Inactivation is blocked by low temperatures or anaerobic conditions—a finding that implicates chemical and, more particularly, oxidative reactions. The mechanism in question could be demonstrated in larval fragments but not in crude or fractionated homogenates.

3. When injected into mature larvae, 1 μg of α-ecdysone loses 50% of its activity in 1 hour and 98% in 8 hours. Lower doses show even briefer "half-lives."

4. The rapid inactivation of ecdysone can account for its low titer in both the blood and tissues. Thus at the "critical period" for puparium formation, the entire larva contains only 2.5 nanograms, corresponding to only 7% of a *Sarcophaga* unit.

5. The evidence points to the accumulation, not of the hormone itself, but the covert biochemical and biophysical effects of the hormone. The covert effects undergo spatial and temporal summation within the target organs and finally discharge the overt developmental response.

6. The role of the blood is to serve, not as a reservoir, but as a pipeline through which ecdysone flows from the ring-gland to its sites of action and swift inactivation.

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LIGHT ORGAN FINE STRUCTURE IN CERTAIN ASIATIC FIREFLIES

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Control of effector activity by nerve has been studied exhaustively in muscle and to a lesser extent in glands and in electric organs. Coordinated light production by congregations of specialized photocytes represents an entirely different kind of effector activity, in which neural control, though long suspected, has only recently been established directly. Thus far it is only in the light organ or lantern of certain fireflies of the beetle family Lampyridae that nerve endings have been demonstrated (Smith, 1963), excitatory volleys in peripheral lantern nerves recorded (Case and Buck, 1963; Buonamici and Magni, 1967; Magni, 1967) and pharmacological evidence of neurosecretory excitation adduced (Smalley, 1965). However, the firefly lantern is a particularly favorable material in which to study excitation-effector coupling because it excels all other photophores in versatility and precision of control and because there exists a large body of detailed information about the biochemistry, physiology and morphology of the light-emitting system.

Recently recordings have been made of the flashing of fireflies of the genus *Pteroptyx* in Bornea and Thailand (Buck and Buck, 1968). These insects can produce flashes lasting only about 30 msec. and can flash repetitively at frequencies of more than 30 per second. Because this is at least three times as sharp a control as in any previously studied firefly, and because electron microscopic studies on species from the tropical orient have not previously been reported, the fine structure of the *Pteroptyx* lantern was studied. For comparison we present also some data for the firefly genera *Luciola* and *Pyrophanes* from the same regions.

MATERIAL AND METHODS

The species studied were *Pteroptyx valida* and *P. malaccae* from Thailand and *Pyrophanes appendiculata* and *Luciola* sp. from Sarawak.[2] The comparative histology of these and other Asiatic species is being reported separately by Mrs. Miriam McLean of our Laboratory, but she has kindly allowed us to introduce the account of ultrastructure with semidiagrammatic sketches made from her slides (Figs. 1 and 2).

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[1] The authors acknowledge with gratitude the extensive technical assistance of Mr. Charles Hanna. (One of us [J. B.] is grateful to The American Philosophical Society [Penrose Fund grant no. 3862] and to the National Geographical Society for travel grants.)

[2] Identifications were kindly made for us by Mrs. L. P. Ballantyne at the University of Queensland, Brisbane, Australia.

Most of our material for electron microscopy was fixed in the field, and of necessity the fixation schedule was modified from the usual, since microscopes, hoods and temperature controls were not available. The abdomens of the specimens were slit open and the whole insects were dropped into 6% glutaraldehyde in Millonig's buffer at ambient (tropical) temperatures. Some specimens were left in glutaraldehyde and others were transferred after an hour and a half to buffer containing a small amount of thymol. They were in transit for from ten days to six weeks before reaching this laboratory, where they were post-fixed in Millonig's OsO_4, dehydrated in alcohol, and embedded in Maraglas. Of these specimens, only *Luciola* showed satisfactory fixation. The other tissues were fixed poorly, but well enough to permit comparisons.

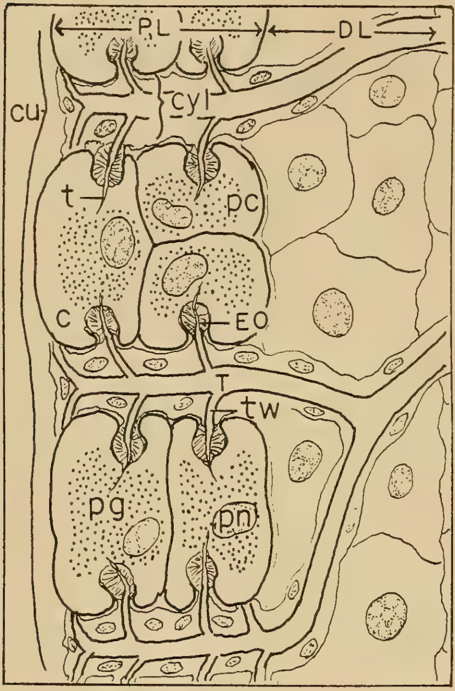

FIGURE 1.

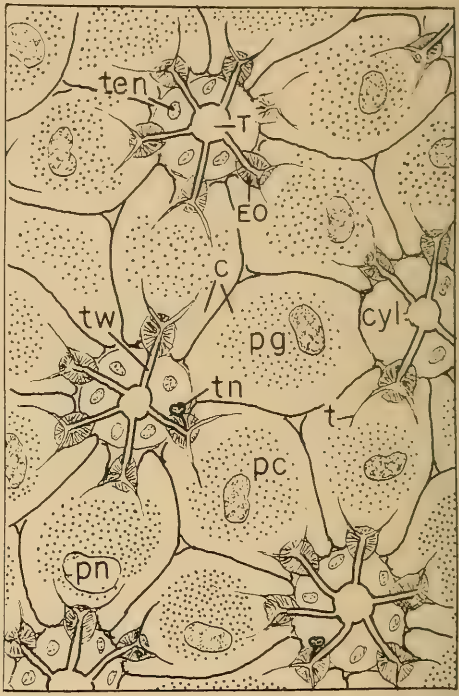

FIGURE 2.

FIGURE 1. Semidiagrammatic drawing of a transverse section through the lantern of *Pteroptyx,* showing portions of the photogenic (PL) and dorsal (DL) layers and three cylinders (cyl) containing tracheal trunks (T). c, cortex of photocyte, pc; EO, end organ; pg, region of photocyte granules; pn, nucleus of photocyte; t, tracheole; tw, tracheal twig; cu, cuticle.

FIGURE 2. Semidiagrammatic drawing of a horizontal section through part of the photogenic layer of a *Pteroptyx* lantern. cyl, cylinder; T, tracheal trunk; tw, tracheal twig; t, tracheole; ten, tracheal epithelial nucleus; tn, tracheolar cell nucleus; EO, end organ; pc, photocyte; c, cortex of photocyte; pg, region of photocyte granules; pn, photocyte nucleus. × 650.

Later, we were very fortunate in obtaining some live specimens of *Pteroptyx* *malaccae*.[3] Lanterns of some of these specimens were fixed in cold 6% glutaraldehyde for one and a half hours, and then treated as above.

General Structure

A surprising finding was the similarity in gross lantern morphology not only among the three Asiatic genera but between them and the common North American genera *Photinus* and *Photuris*. As seen by light microscopy (Figs. 1 and 2) the organs consist of a photogenic layer usually two cells thick, lying immediately internal to the transparent ventral abdominal cuticle and backed by a dorsal ("reflector") layer several cells thick. Running vertically through both layers are tracheal trunks. In the photogenic layer each trunk runs within a wide tunnel or cylinder into which it gives off a multitude of short tracheal twigs. In horizontal

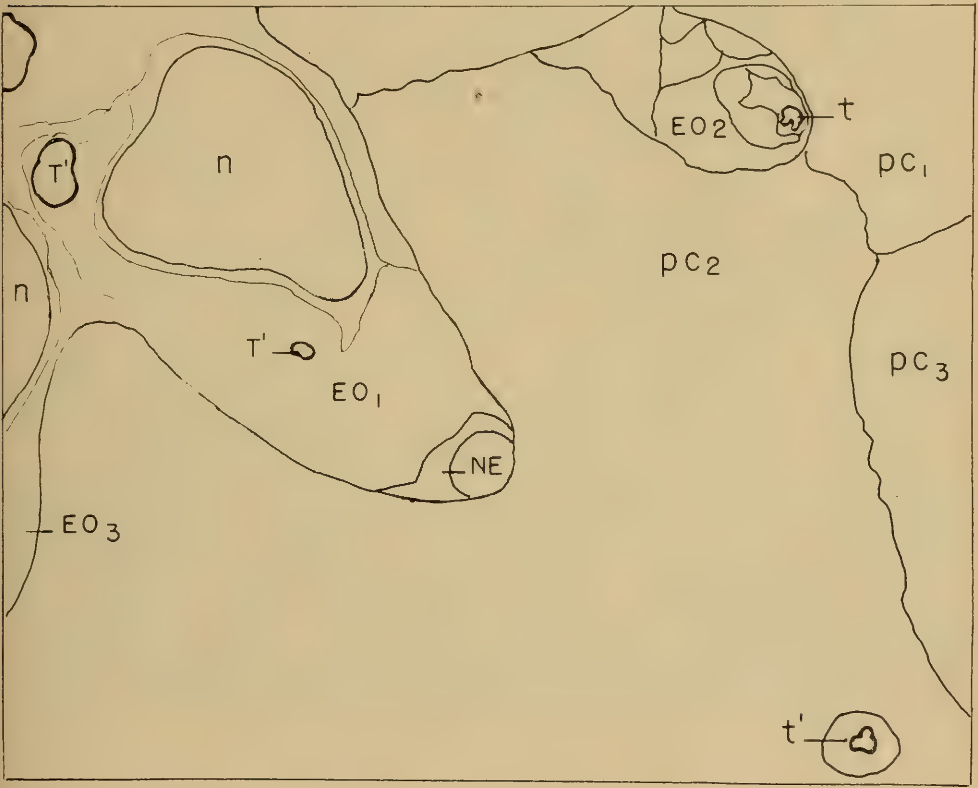

FIGURE 3A. Outline of cell boundaries in Figure 3B as an aid to orientation. EO_1, EO_2, and EO_3, end organs; pc_1, pc_2, and pc_3, photocytes; T′, tracheal twig; t and t′, tracheoles; n, twig cell nuclei. NE, terminal portion of nerve.

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[3] Live *Pteroptyx malaccae* were obtained by Dr. Douglas Gould of the Walter Reed Army Medical Center unit of the Seato Medical Research Project, Thailand, and brought to Bethesda by Dr. Robert Gordon of our Institute.

section (Fig. 2), it is seen that these cylinders are arranged in triangular symmetry, each surrounded by a radial rosette of polyhedral photocytes. At the periphery of the cylinders each twig terminates in a minute "tracheal end organ" which protrudes into the body of a photocyte. From the end organs issue delicate tracheoles which can be followed but a short distance as they penetrate an external "differentiated zone" or cortex, staining differently from the interior cytoplasm. In the Asiatic species the number of tracheoles originating from a single tracheal twig is apparently always two, in contrast to the three or four of *Photinus* and the two or three (Smith, 1963) or three to seven (Kluss, 1958) reported for *Photuris*.

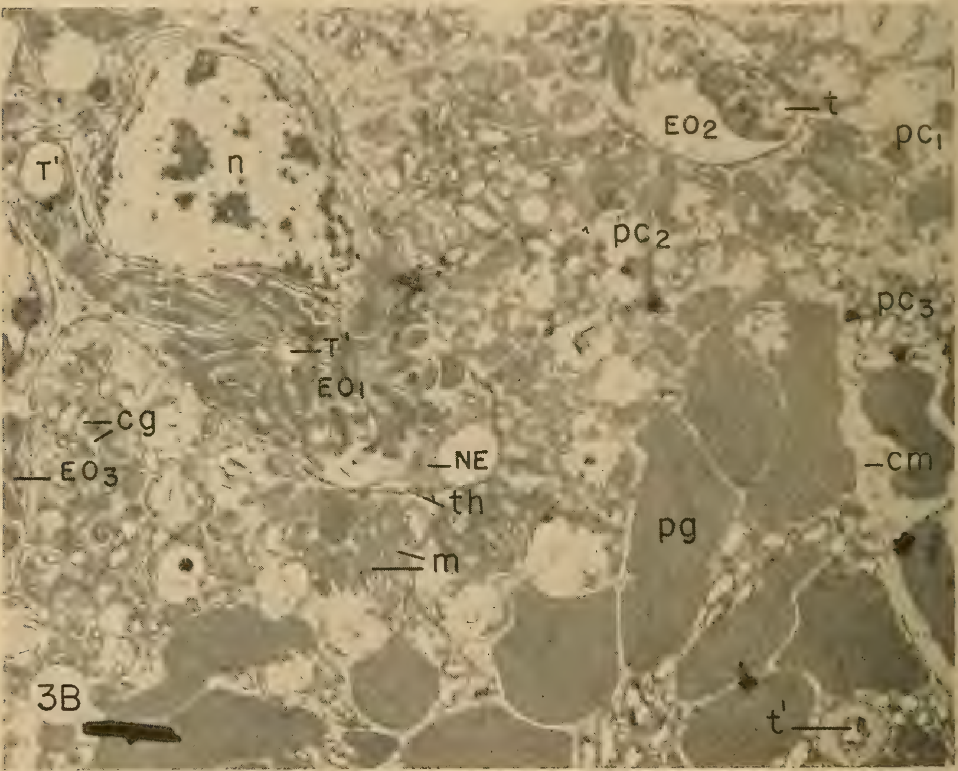


FIGURE 3B. Low power electron micrograph of region of contact between a cylinder and the photogenic layer, showing parts of three end organs (EO_1, EO_2, EO_3) and of three photocytes (pc_1, pc_2, pc_3). T′, tracheal twig; t, tracheole in tracheolar cell; t′, section of tracheole within photocyte cytoplasm enclosed by sheath of tracheolar cell cytoplasm and tracheolar cell membrane; n, nucleus of tracheal twig cell; NE, terminal portion of nerve; th, electron-dense thickenings along contact of end organ and photocyte membranes; m, photocyte mitochondria grouped around end organ; cg, clear-centered granules in cortical region of photocyte; pg, photocyte granule; cm, cell membrane of photocyte. *Luciola* sp. × 6600.

Previous electron microscopic studies of *Photinus* (Beams and Anderson, 1955) and *Photuris* (Kluss, 1958; Smith, 1963) have shown that the tracheal "end cell" is actually a highly complex structure consisting of an inner tracheolar cell ensheathing the tracheoles and a concentric outer cell enclosing the tracheal twig and its branching point and enveloping the tracheolar cell. Since this outer cell is very different from the usual tracheal end cell of insect tissues, we shall call it the "twig cell." Also in the interest of clarity we shall adopt Kluss' "tracheal end organ" to describe the entire complex, and we shall use "cortex" instead of "differentiated layer" or zone for the specialized peripheral zone of cytoplasm which is seen at all external or free faces of the photocytes—*i.e.*, where a photocyte abuts on the dorsal layer, on a cylinder, or on the ventral epithelium, but not where two photocytes touch.

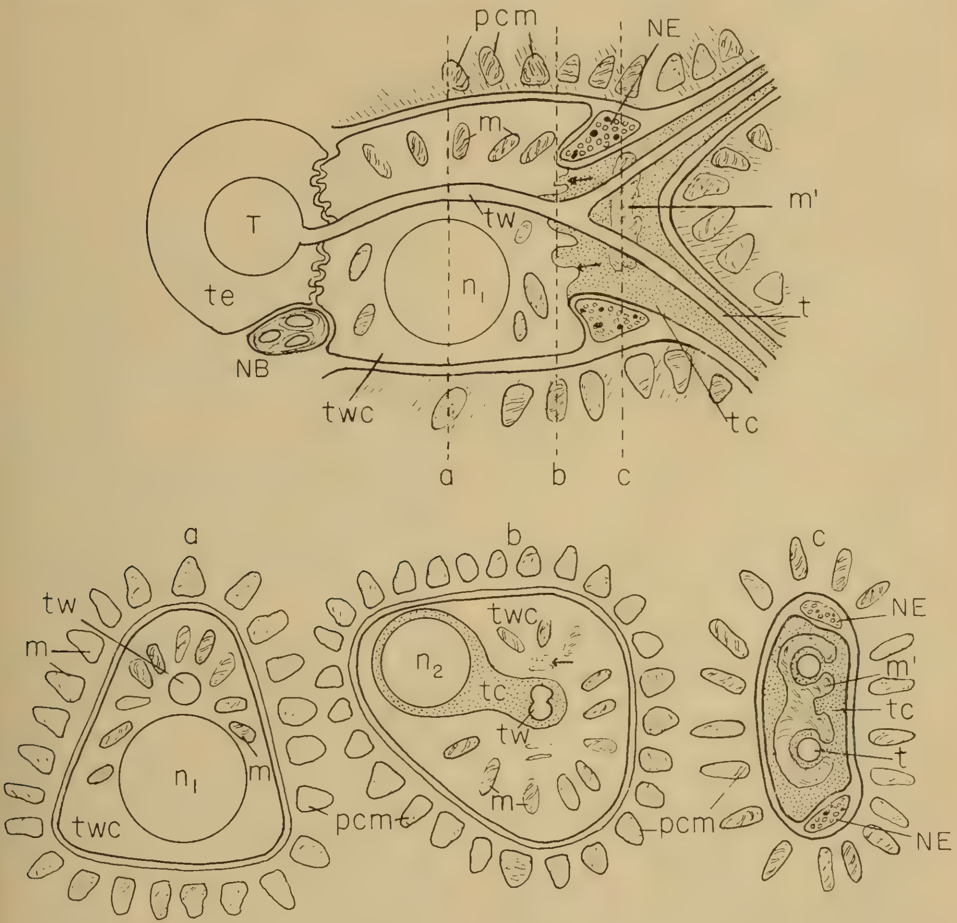
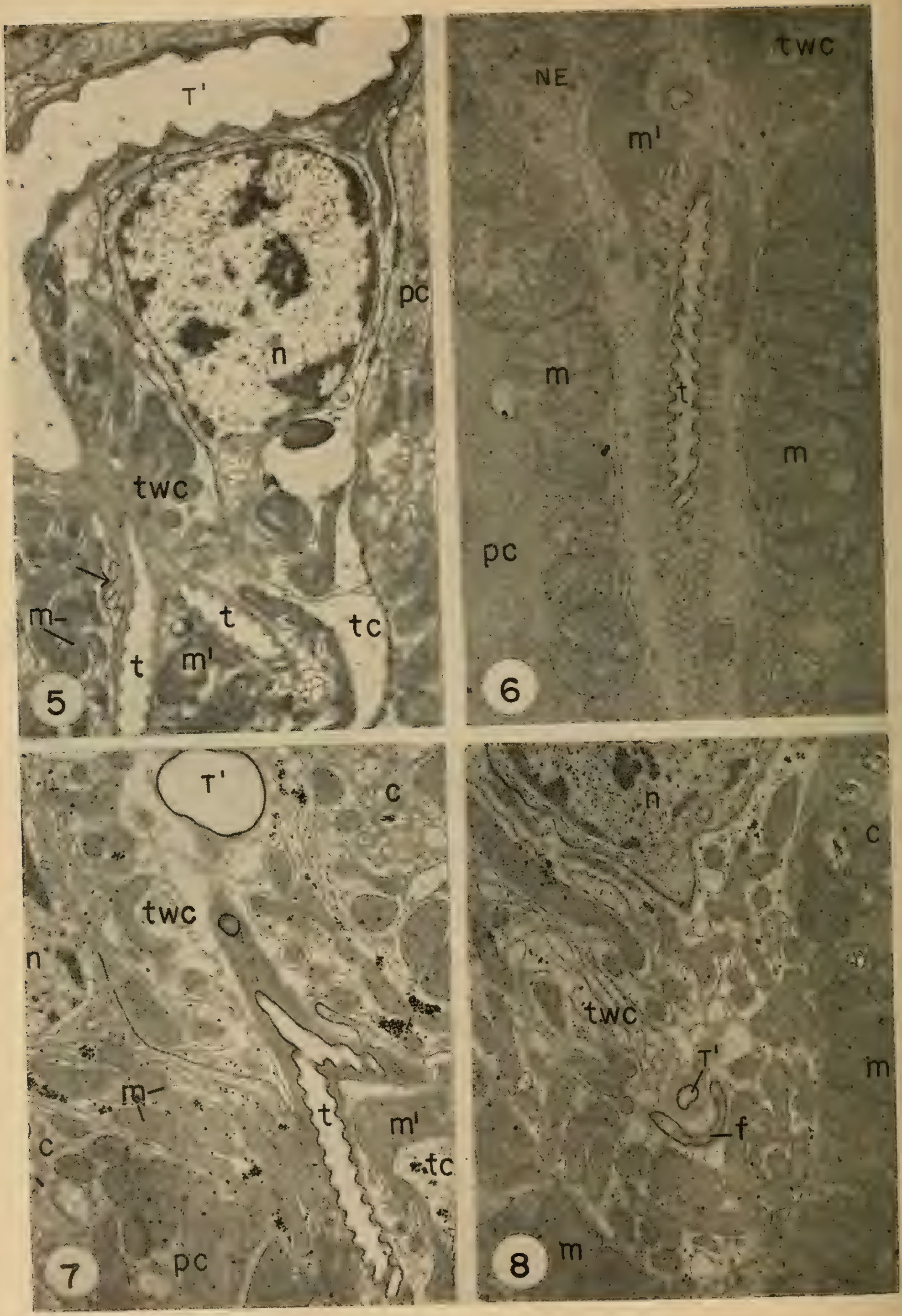


FIGURE 4. Diagram of an end organ to show the relationship of its components (twig cell, twc; tracheolar cell, tc; nerve endings, NE) to each other and to the photocyte. Transverse sections taken at the levels indicated by broken lines are represented in a, b, and c. T, trachea; tw, tracheal twig; t, tracheole; te, tracheal epithelial cell; m, mitochondria of twig cell; pcm, mitochondria of photocyte; m′, large mitochondrion enveloping origins of tracheoles; NB, nerve branch approaching end organ; n_1, nucleus of twig cell; n_2, nucleus of tracheolar cell.


T'
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FIGURES 5–8.

At the electron microscope level Asiatic lantern topography is also quite similar to that in *Photinus* and *Photuris*. We shall accordingly not describe the fine structure in general but concentrate on the several important structural differences between the two groups of species. Figure 3B gives a low power view of the region of contact between cylinder and photogenic layer. Parts of two end organs and of three photocytes are shown. The protrusions of the photocytes, which interdigitate tightly with the end organs, consist exclusively of cortical cytoplasm containing closely packed mitochondria and clear-centered granules. A nerve terminal shows near the distal tip of the end organ close to the twig cell-photocyte apposition plane. (The clear space under the NE label is an artifact.) The line of apposition between end organ and photocyte is marked by many electron-dense thickenings which are lacking on stretches of membrane between photocytes. A section of a tracheole is seen within the photocyte cytoplasm still accompanied by a sheath of tracheolar cell cytoplasm and the infolded photocyte membrane (t′).

TRACHEAL END ORGAN

Because of its presumed involvement in excitation of luminescence (see below), the treacheal end organ cannot be discussed in isolation from the structures with which it makes direct contact, particularly the photocyte.

The end organs of *Pteroptyx*, *Pyrophanes* and *Luciola* conform in general to the pattern described in detail by Smith (1963). The relationship of its parts can be followed by reference to the diagram (Fig. 4). The twig cell membrane is thrown into profuse finger-like radial folds in which lie elongated mitochondria (Figs. 5; 7, m). The cell membrane also interdigitates distally with the tracheolar cell membrane (arrows, Figs. 4 and 5). However, the Asiatic species differ from *Photuris* in a number of important respects, chief among which is the point of termination of the nerves. In *Photuris*, the ends of the finest peripheral nerves lie between the tracheolar and twig cells and the neuronal membrane is apposed equally to the membranes of these two cellular components of the end organ. It is thus separated from the photocyte by the twig cell cytoplasm and membranes, a distance of usually not less than a micron. In the oriental species the bell-shaped twig cell overlaps only the proximal portion of the tracheo-

FIGURE 5. Longitudinal section through an end organ of *Luciola* sp. The tracheal twig (T′) curves around the nucleus (n) of the twig cell (twc) before branching into two tracheoles (t). A special large lobed mitochondrion (m′) in the tracheolar cell (tc) envelops the tracheoles just beyond their origin. Twig and tracheolar cell membranes interlock (arrow). pc, photocyte; m, mitochondria of twig cell. × 11,400.

FIGURE 6. Section through an end organ of *P. malaccae* in the plane of one tracheole (t). Note especially the lobes of the large tracheolar cell mitochondrion (m′) protruding into cuticular folds of the tracheolar wall, and the array of photocyte mitochondria (m) along the tracheole. twc, twig cell; NE, nerve ending; pc, photocyte. × 11,400.

FIGURE 7. Longitudinal section through an end organ of *P. malaccae* showing the origin of the two tracheoles (t). T′, tracheal twig; twc, twig cell; tc, tracheolar cell; m′, large mitochondrion of tc; n, nucleus of tc; c, cortex of photocyte (pc); m, mitochondria of twig cell. × 11,400.

FIGURE 8. Transverse section through an end organ of *P. malaccae*, proximal to branching point of tracheal twig (T′). n, twig cell nucleus; m, mitochondria, and c, cortex, of photocyte; f, fold of tracheolar cell cytoplasm. × 11,400.

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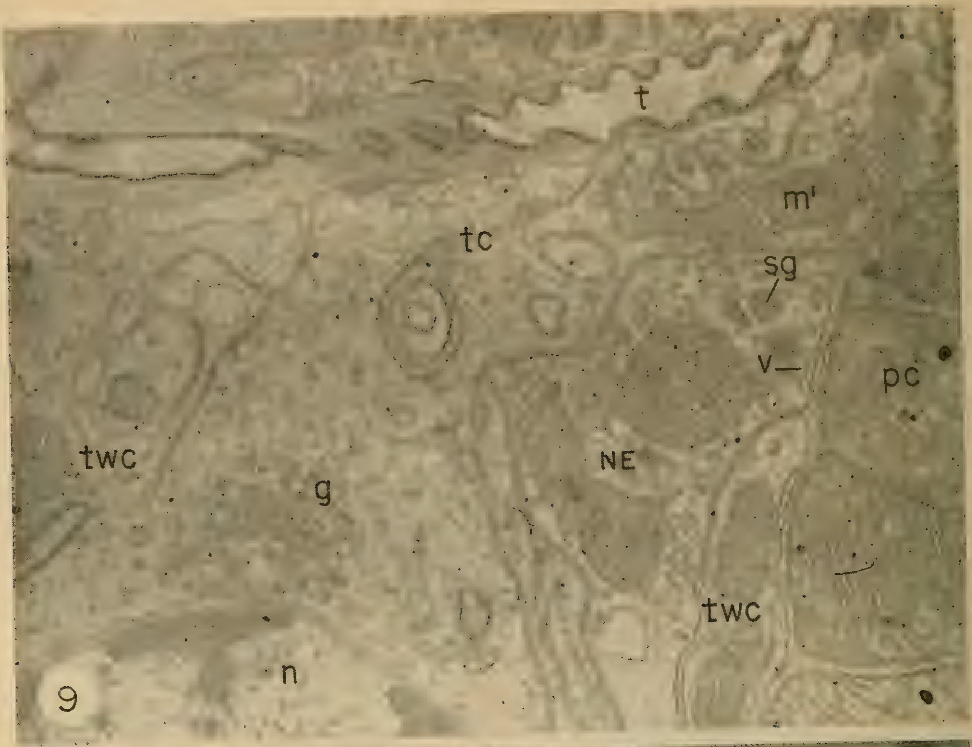
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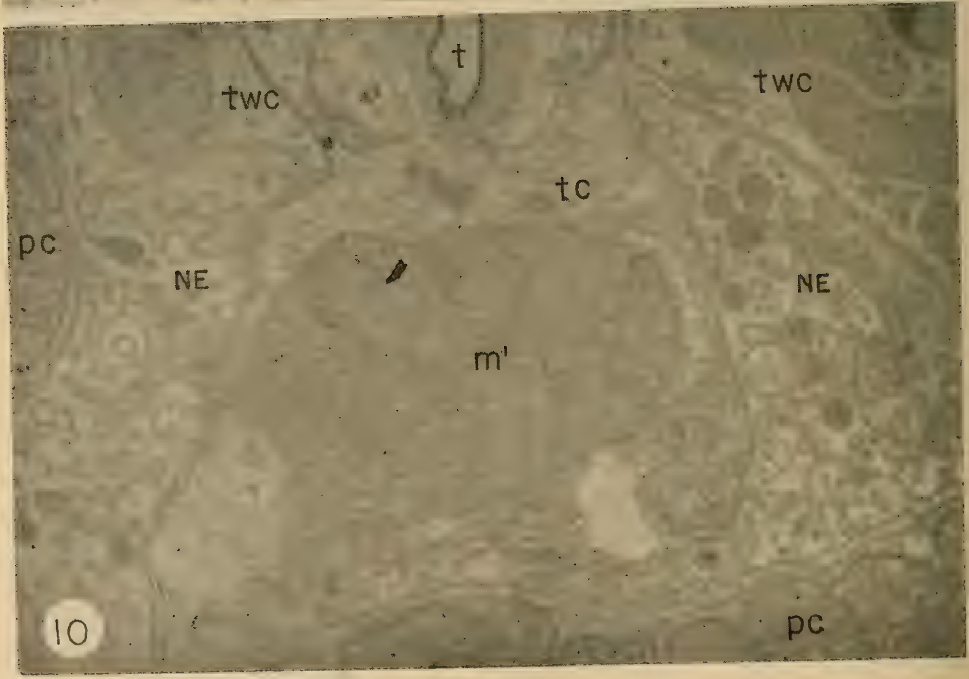
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FIGURES 9–10.

lar cell, rather than covering it completely. Consequently the nerve terminals can and do make contact not only with the tracheolar cell and twig cell membranes but with the photocyte membrane as well. A very careful search of several hundred micrographs failed to disclose any structure analogous to the tight junction or subsynaptic web of the neuromuscular junction of vertebrates. The neuronal contact with the membrane of the photocyte appears to be somewhat less close than that with the cells of the end organ (Figs. 9–12).

The end organs of Asiatic fireflies differ markedly from their American counterparts in having a single, very large, branched mitochondrion wrapped around the two tracheoles just distal to their origin from the tracheal twig (Figs. 11, 15, m′). There seems to be a tendency for the nerve terminal to be very intimately associated with this mitochondrion, both laterally and distally (Figs. 9–12, 15).

As in *Photinus* and *Photuris* the end organs are closely associated with the photocytes. Fingers or lobes of cortical photocytoplasm interdigitate with end organs, and the tracheoles with their sheaths of tracheolar cell cytoplasm push in between each pair of contiguous photocytes. Smith (1963) showed that the mitochondria of the cortex tend to congregate at the photocyte membrane. This effect is much more pronounced in the Asiatic fireflies, in which the mitochondria are larger, tend to a conical shape with base against membrane, and are often crowded tightly together in lateral contact (Figs. 6 and 15). This orientation is particularly striking along the proximal portions of the tracheoles. As the latter penetrate beyond the cortex of the photocytes, they are often surrounded by a looser group of more nearly spherical mitochondria, which may sometimes be replaced by photocyte granules.

TRACHEOLES

Whatever they may be in other insects and other tissues, firefly lantern tracheoles appear to be more than merely taenidialized tubes for gas conduction. We shall therefore understand the term to include not only the cuticular lining but also the cytoplasmic sheath from the tracheolar cell which covers the intima throughout its course.

The tracheoles in the Asiatic fireflies show the same minutely villous honeycombed outpocketings of the sclerotized lining (Fig. 14) as they do in *Photuris* (Smith, 1963), and the same sheath of cytoplasm, in which mitochondria are sometimes intercalated between the luminal protrusions (Fig. 11, arrows). Whereas *Photuris* tracheoles follow an exclusively extracellular course between the apposed cell membranes of contiguous photocytes, the *Pteroptyx* tracheoles penetrate deeply into the cytoplasm, sometimes even approaching the photocyte nucleus

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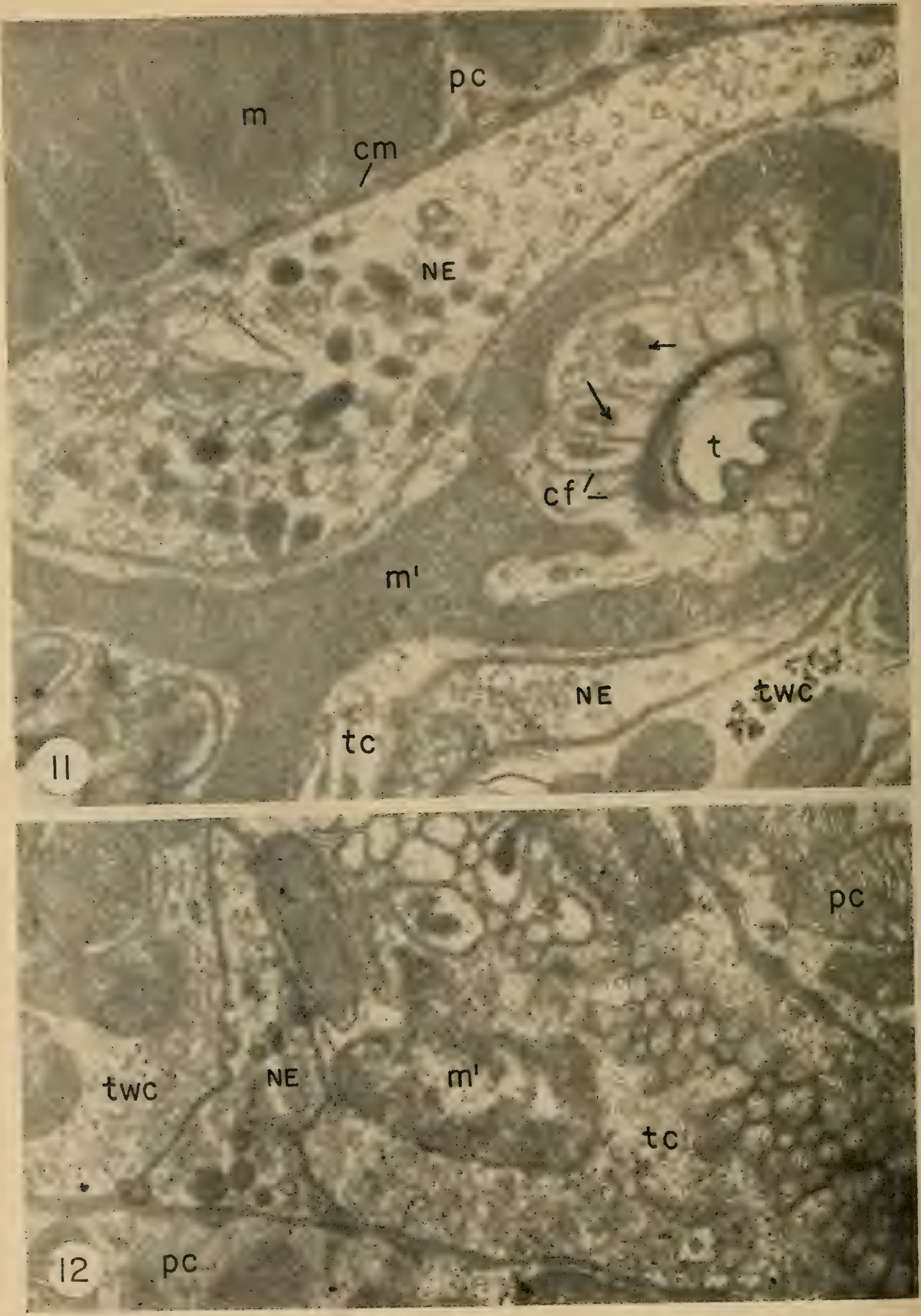
FIGURE 9. Section through an end organ of *P. malaccae,* parallel to one tracheole (t), showing terminal portion of nerve (NE) in contact with three types of cells (twc, tc, pc). m′, special tracheolar cell mitochondrion; n, nucleus of tracheolar cell; g, Golgi body; v, vesicle; sg, granule. × 34,000.

FIGURE 10. Section through end organ of *P. malaccae,* showing three contacts of massive terminal portion of nerve (NE). t, tracheole; twc, twig cell; tc, tracheolar cell; pc, photocyte; m′, special tracheolar cell mitochondrion. × 34,000.


pc
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FIGURES 11–12.

(Fig. 13). The penetration is not truly intracellular, since a sheath of the photocyte membrane accompanies the tracheoloar intrusion (Fig. 13), but contact with the photocyte interior is otherwise intimate.

The lumen of the tracheoles seems everywhere to have a diameter of about 0.3 μ, and the cytoplasmic sheath, once free of the tracheolar cell body, does not taper appreciably. The question arises as to whether the tracheoles end blindly or anastomose with tracheoles from an adjacent cylinder as claimed by Buck (1948) on the basis of light microscopic evidence from *Photinus*. Electron microscope preparations leave the matter still moot. Beams and Anderson (1955) saw no evidence of anastomosis in *Photinus pyralis;* Kluss (1958) states that *Photuris* tracheoles do run continuously from one cylinder to the next; Smith (1963) does not mention tracheole termination. Our own preparations have not disclosed anything identifiable as an ending, but neither have they provided anything like the continuous several micron-long stretch of tracheole midway between cylinders that would be needed to support tracheolar anastomosis with any confidence.

PHOTOCYTE GRANULES

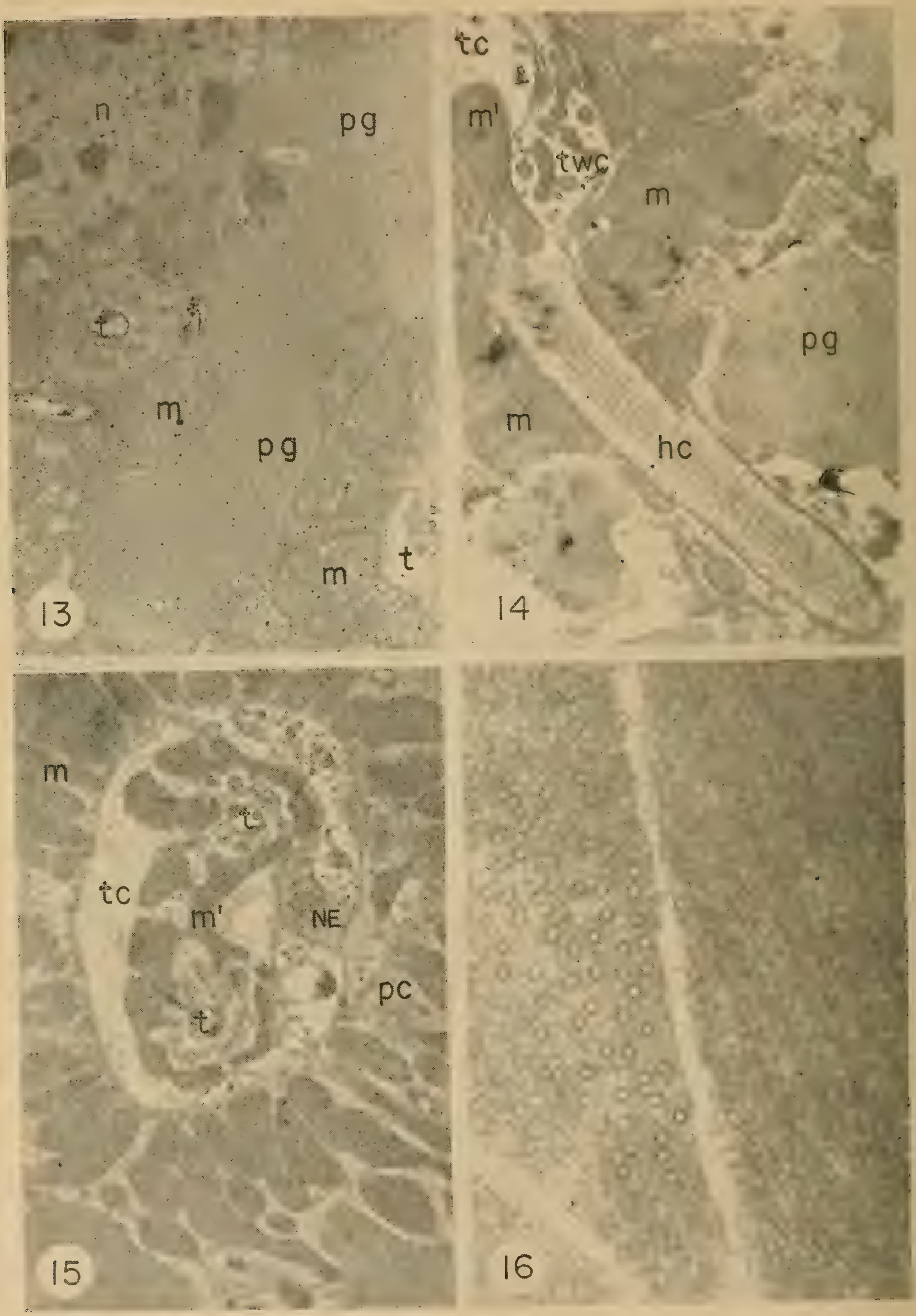
The large granules that are the most prominent inclusion of the interior cytoplasm of photocytes are also the most enigmatic. For about a century they have been referred to as "photogenic granules" or the equivalent without a scrap of direct evidence that they are involved in light emission. In our oriental species they tend to be elongated, rather than spherical, and to be larger (4.5×2.5 μ) than in *Photinus* and *Photuris*. We have only rarely seen single interior vacuoles or vesicles, sometimes connected to what looks like a slender duct, that were occasionally seen in *Photuris* photocyte granules by Smith (1963). Instead we find heavy concentrations of typical microtubules, 230 Å o.d., 150 Å i.d. (Fig. 16). There is some evidence of the tubules curving in conformation with the contour of the granule, but no real hint as to what their function may be.

INTERCELLULAR CONTACT

Smith described quite striking "adhesion plates" connecting the surface membranes of contiguous photocytes at intervals. We have not observed such structures in our material. However, we find rather regularly spaced triangular nodes of enhanced electron density along the line of contact between end organ and photocyte. In the domain of tracheolar cell-photocyte membrane contact the nodes appear to be about twice as numerous as in the region of twig cell-photocyte contact (Figs. 11, 12).

FIGURE 11. Transverse section through end organ of *Luciola* distal to branching of tracheal twig (*cf.* Figure 4c). Nerve endings (NE) make contact with twig cell (twc), tracheolar cell (tc) and photocyte (pc), and also follow the contours of the large tracheolar cell mitochondrion (m′). t, tracheole; m, mitochondria of photocyte clustered around end organ. Note protrusions of tracheolar cell mitochondrion into cuticular folds of tracheolar wall (arrows). × 54,000.

FIGURE 12. Longitudinal section through end organ of *Luciola* showing contact of nerve endings (NE) with twig cell (twc), tracheolar cell (tc), and photocyte (pc). m′ = special tracheolar cell mitochondrion. × 34,000.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Figures 13–16.

Discussion

Comparative histological data strongly indicate that tracheal end organs are present in all firefly species able to produce sharp flashes and absent in species able only to emit a long-lasting glow (Buck, 1948). This information, when combined with the facts that nerves terminate in the end organs (Smith, 1963) and that the light emitted by the organ seems to come from the region of cylinder-photocyte contact (review in Buck, 1966), strongly indicates that the end organ-cortex region is in some sense a neuroeffector junction. It is by no means clear, however, that the junction conforms to the pattern seen in neurally excitable muscle or gland or in interneural synapses.

The evidence for neurosecretory excitation of firefly luminescence has rested primarily on the occurrence of neuroeffector facilitation during repetitive electrical stimulation (Buck and Case, 1961) and on the close morphological similarity, pointed out by Smith, between the two types of inclusions seen in the peripheral nerve terminals of the lantern and the "neurosecretory droplets" and "synaptic vesicles" of the vertebrate neurohypophyseal junction (Palay, 1956). The case has recently been strengthened by direct evidence of involvement of noradrenalin in excitation (Smalley, 1965). Nevertheless, neither in Smith's preparations of the *Photuris* lantern nor in ours from the Asiatic species does the membrane of the nerve terminal show the tight membranal junctions with another cell membrane and sub-synaptic web which are characteristic, for example, of many neuroeffector or neuronal synapses (De Robertis, 1967).

The discovery that the nerve terminals in *Pteroptyx* and its Asiatic neighbors make direct contact with the presumed effector cell of the lantern, the photocyte, only partly relieves the difficulty that, as in *Photuris*, they seem also to be closely associated with both the tracheolar and twig cell membranes. A conceivable role of tracheoles in excitation as well as in gas conduction is suggested by their profuse distribution, deep inter- and intra-cellular penetration, and the special structure of their enveloping cytoplasm. On the whole, the present ultrastructural evidence leaves the whole question of the triggering of bioluminescence in the firefly lantern in an unsatisfactory conceptual state, but certainly indicates that the junction is of a quite new type.

Figure 13. Portion of a photocyte of *P. malaccae* showing two sections of tracheole (t) within the cytoplasm, one very close to the nucleus (n) of the photocyte. Note that the membranes of tracheolar wall and of photocyte can both be seen. Mitochondria (m) cluster around tracheole. Photocyte granules (pg) contain numerous microtubules in cross section. × 11,400.

Figure 14. Part of an end organ and tracheole of *P. valida*, poorly fixed, but demonstrating the honey-comb structure (hc) of the tracheolar wall. tc, tracheolar cell; twc, twig cell; m′, tracheolar cell mitochondrion; m, photocyte mitochondria; pg, photocyte granule. × 11,400.

Figure 15. Section through a tracheolar cell of *Luciola*, beyond the overlap of the twig cell (*cf.* Fig. 4c). t, tracheole; tc, tracheolar cell; m′, tracheolar cell mitochondrion; NE, nerve ending; pc, photocyte with many mitochondria (m) radiating around tracheolar cell. × 11,400.

Figure 16. High magnification electron micrograph of a portion of two photocyte granules of *Pyrophanes*, showing cross-sections and longitudinal sections of numerous microtubules. × 80,000.

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REPRODUCTIVE CYCLE OF THE SURF CLAM, SPISULA SOLIDISSIMA, IN OFFSHORE NEW JERSEY

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Atlantic surf clams, *Spisula solidissima* (Dillwyn), were collected from offshore New Jersey during a three and one-half-year period to obtain their gonads for histological treatment in a study of the annual reproductive cycle. Documentation of the annual reproductive cycle on the basis of gonad distention (Westman and Bidwell)[1] and excision of gametes (Allen, 1951, 1953; Schechter, 1941) is incomplete and fragmentary. Neither gonad distention nor excision of gametes assesses the events of gamete development within the gonad. Also, parasites are a possible source of error if the technique of observing gonad distention is used. I have seen infected surf clams with distended gonads when those of uninfected individuals were shrunken. Gonad color has been used as an indicator of sex and gonad development of the sea scallop, *Placopecten magellanicus* (Posgay and Norman, 1958; Merrill and Burch, 1960). The color of surf clam gonads containing mature sex products, however, is not consistent. Ovaries of females are sometimes pink, but may also be white, as are the testes (Schechter, 1941).

Periodic histological examinations of gonad tissue during several successive years has been a valuable method of determining the annual frequency and duration of the reproductive cycle in many marine invertebrates (Giese, 1959). The reproductive cycles of the soft-shelled clam, *Mya arenaria* (Pfitzenmeyer, 1962, 1965; Shaw, 1962, 1965; Ropes and Stickney, 1965) have been recently established by describing the cytological events within the gonad. Such events within the gonads of surf clams from an area of intensive fishing off the New Jersey coast have been related to water temperature. The present study of seasonal gonad development was undertaken to determine the annual frequency and duration of spawning—information basic to understanding larval occurrence and time of setting of juvenile surf clams.

MATERIALS AND METHODS

Surf clam gonads were collected from port landings (1962 and 1963) and commercial and research vessels (1964 and 1965). In all years, samples were taken as soon as practicable after the clams were removed from the water, because the surf clam readily reacts to artificial spawning stimuli (Stickney, 1963) and some released sex products soon after capture. All samples were from the vicinity of Barnegat Lightship and were caught by hydaulic dredges operating at depths of 18 to 32 meters. A sample was taken monthly during the winter and biweekly during the spring, summer, and fall. Each sample comprised 25 live clams with shells 120 mm. long or longer. The portion of the gonad ventrad to the heart was removed after fixation of the entire visceral mass in Bouin's solution, thereby preventing the loss of eggs and sperm, and minimizing the possible variability in development within a single gonad. Standard histological techniques were employed to prepare sections 7 μ thick for staining with Delafield's hematoxylin and eosin.

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[1] Westman, J. R., and M. H. Bidwell, 1946. The surf clam. Economics and biology of a New York marine resource. State of New York Conservation Dept. Unpublished manuscript on file at Bureau of Commercial Fisheries Biological Laboratory, Oxford, Maryland 21654.

After microscopic examination of the section, each specimen was assigned to a category of gonad development. The proportions of clams in each category, regardless of sex, were recorded for individual samples and the samples were combined each month to simplify presentation. In graphic presentations of the results, the combined data are plotted at a date midway between the sample dates.

The possible influence of temperature on gonad development and spawning was considered. Surface and bottom sea-water temperatures at Barnegat Lightship were obtained from Chase (1965, 1966) for 1962 and 1963 and from unpublished records for 1964 and 1965.

Anatomy and Spawning

Male and female surf clams are distinguishable by microscopic examination of the gonads because they are unisexual or dioecious (Coe, 1943). Hermaphroditism is rare and then is apparently a developmental accident (Ropes, 1966).

The gross features of a surf clam's reproductive anatomy include anastomosing gonadal alveoli which lie ventrad to the pericardial chamber and overlay the dark brown digestive gland or liver mass, and surround the coiled intestine. In the distended gonads before spawning, the root-like alveoli are crowded close to each other and are filled with "late ripening" and "ripe" sex cells; their connective tissue walls are stretched thin; and they occupy much of the visceral mass. After spawning the alveoli contract, their walls thicken, and mesenchyme is interspersed between them; the dark brown digestive gland is then the most prominent organ and the visceral mass is flaccid.

The alveoli lead into a pair of gonoducts, each of which has an enlarged portion that serves as a reservoir for ripe sex cells before spawning (Stickney, 1963). The gonoducts terminate at the genital apertures on minute papillae that protrude from the surface of the posterior foot retractor muscle on each side of the visceral mass.

The passage of water for respiration and digestion conducts sex cells from the clam into the surrounding water during spawning. Water drawn through the incurrent siphon flows into the infrabranchial chamber, around the gills, past the genital papillae, into the epibranchial chamber, and then out through the excurrent siphon. Spawning is often accompanied by rhythmic contractions of the adductor muscles which pull the two valves together. Gametes are either emitted into the water by the muscular activities or flow out. Ovocyte maturation follows spawning and fertilization (Costello *et al.*, 1957; Stickney, 1963) but meiosis occurs in the gonads of male surf clams and functional sperm are developed before spawning.

CATEGORIES OF GONAD CONDITION

In this study the more or less continuous reproductive process was divided into five phases as described below. The demarcation between phases is not sharp—the divisions are convenient rather than natural.

Female gonads

1. *Early active phase*

In the early active phase ovogonia appear at the periphery and are embedded in the alveolar walls, often before the gonads are completely empty of ripe ovocytes. The round and oval nuclei of ovogonia contain distinctly basophilic nucleoli and the nucleoplasm has thin, irregularly shaped, dark-staining threads (Fig. 1). Cytoplasm surrounds the nuclei but is indistinct and of irregular shape. The ovogonia are possibly undergoing the pre-meiotic stages similar to those described for the desert snail, *Eremina desertorum* (Fahmy, 1949) and the soft-shelled clam, *Mya arenaria* (Coe and Turner, 1938). The alveolar walls are not completely contracted and lumina are evident in most gonads.

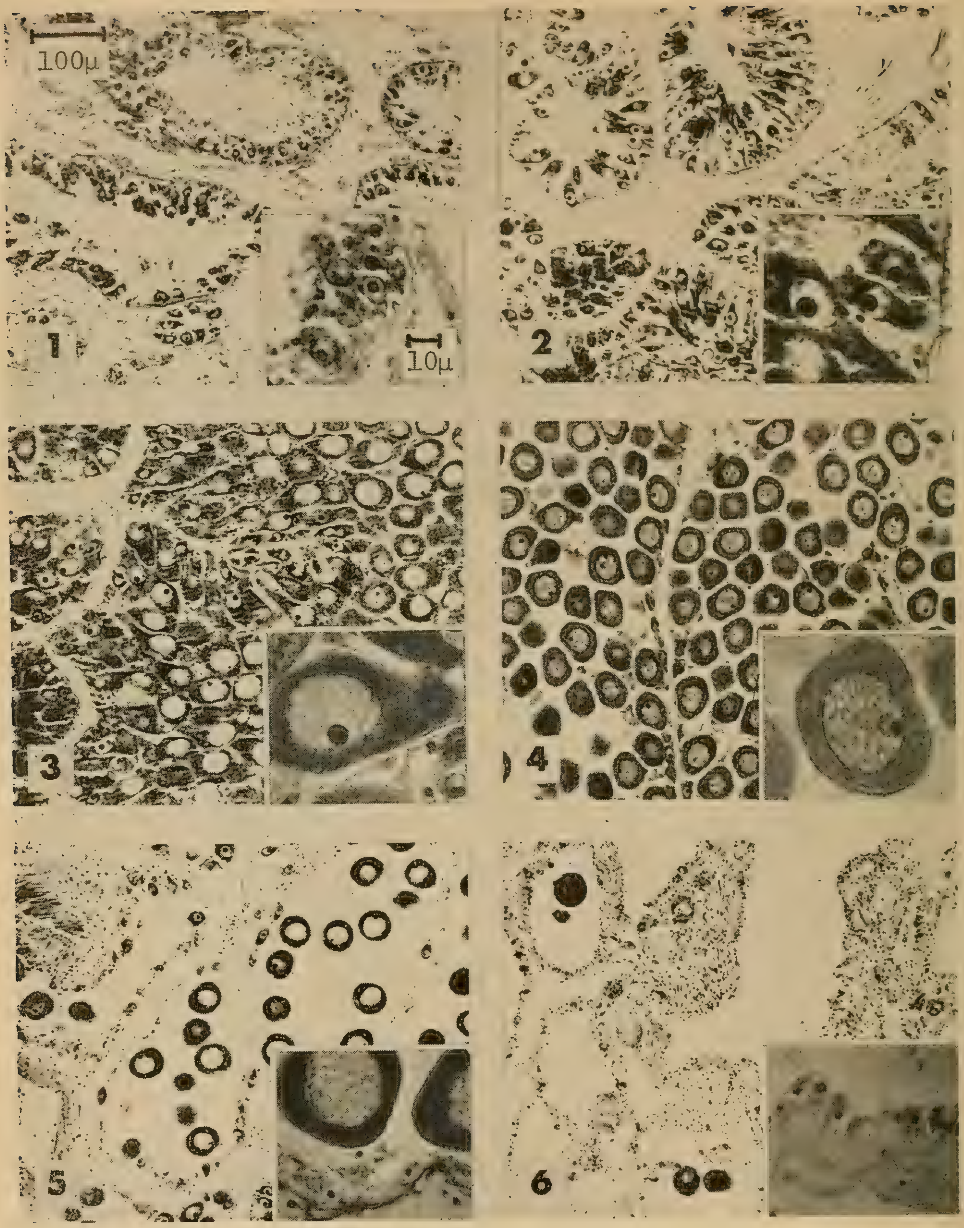
The early ovocytes also have mostly round and oval nuceli containing basophilic nucleoli, but the nucleoplasm is homogeneous and almost clear (Fig. 2). The ovocytes lie at the periphery of the alveolar walls and the cytosome extends into the lumen of the alveolus, where a rounded cap is formed. The shape of the ovocytes may be square, triangular, cylindrical, or hemispherical. All of these ovocytes are attached to the basement membrane by a stalk. The diameters of the nuclei range from 5 to 20 μ and average 11.1 μ.

2. *Late active phase*

Ovocytes in the late active phase are larger than early ovocytes and many are round (Fig. 3). The nuclei contain basophilic nucleoli and the nucleoplasm is homogeneously granular. The nuclei, surrounded by cytoplasm, are the most conspicuous characteristic of the cells. Some ovocytes are attached to the basement membrane of the alveoli by a thin stalk, but most are free in the lumina. The basement membrane is thin—apparently due to the increased volume of cells within the alveoli. The diameters of the nuclei range from 14 to 32 μ and average 24.1 μ.

3. *Ripe phase*

In ripe clams, ovocytes are free in the lumina of the alveoli. The vitelline membrane contains the cytoplasm and a large nucleus (Fig. 4). These ovocytes are very similar to those in the late active phase except for the presence of prominent amphinucleoli (Allen, 1953). Each amphinucleolus consists of an almost transparent, granular nucleolus and a small opaque, basophilic nucleolinus. Ripe gonads typically have a dense appearance because the alveoli are crowded together and are filled with large ovocytes. The diameters of the nuclei range from 22 to 33 μ and average 28.5 μ.



Figures 1–6.

Figure 1. Section of gonad tissue from a female surf clam, early active phase of the first annual reproductive cycle. All of the large photomicrographs are reproduced at the same magnification and a scale (100 μ) is provided. The small photomicrographs inserted in the lower right-hand corner of each figure are enlargements of portions of the gonad all at the same magnification and a scale (10 μ) is provided. Collection date: 21 January 1964.

4. *Partially spawned*

A few large, ripe ovocytes are free in the lumina of some alveoli of partially spawned clams; other alveoli are completely void of ripe ovocytes (Fig. 5). Ovogonia and early ovocytes appear in the contracted and thickened alveolar walls. The spaces between alveoli contain mesenchyme and the whole gonad has a loose, flaccid appearance. In some clams, ripe ovocytes are being cytolyzed.

5. *Spent*

In typical spent clams, the open lumina of alveoli are usually void of ripe ovocytes. The thickened alveolar walls contain ovogonia and early ovocytes (Fig. 6). Mesenchyme occurs in the spaces between the loosely arranged alveoli.

Male gonads

The criteria for the five categories of gonad condition in the male are presented below:

1. *Early active phase*

Clams in the early active phase contain cells undergoing the initial meiotic stages of development. Darkly stained spermatogonia are in the thickened alveolar walls (Fig. 7). Primary spermatocytes about 3 to 4 μ in diameter proliferate toward the lumina from a close contact with the alveolar walls and form single cell columns (Fig. 8). Their large, round and oval nuclei are surrounded by thin irregularly shaped cytoplasm. Two basophilic nucleoli within some cells indicate that cell division is taking place. The nucleoli are less basophilic and smaller than those in the earliest ovocytes.

2. *Late active phase*

The remaining spermatogenic stages are seen in clams in the late active phase. Secondary spermatocytes of about 2 to 3 μ in diameter with dark basophilic chromatin threads that nearly fill the nuclei are numerous and the surrounding cytoplasm is irregular in shape and very indistinct (Fig. 9). Small and strongly basophilic spermatids measuring about 1.5 μ in diameter form dense masses near the centers of the alveoli. A transformation of the spermatids results in the appearance of sperm in the lumina. The sperm form weak columns with the tails oriented toward the centers of alveoli. The sperm heads are about 1 μ in diameter.

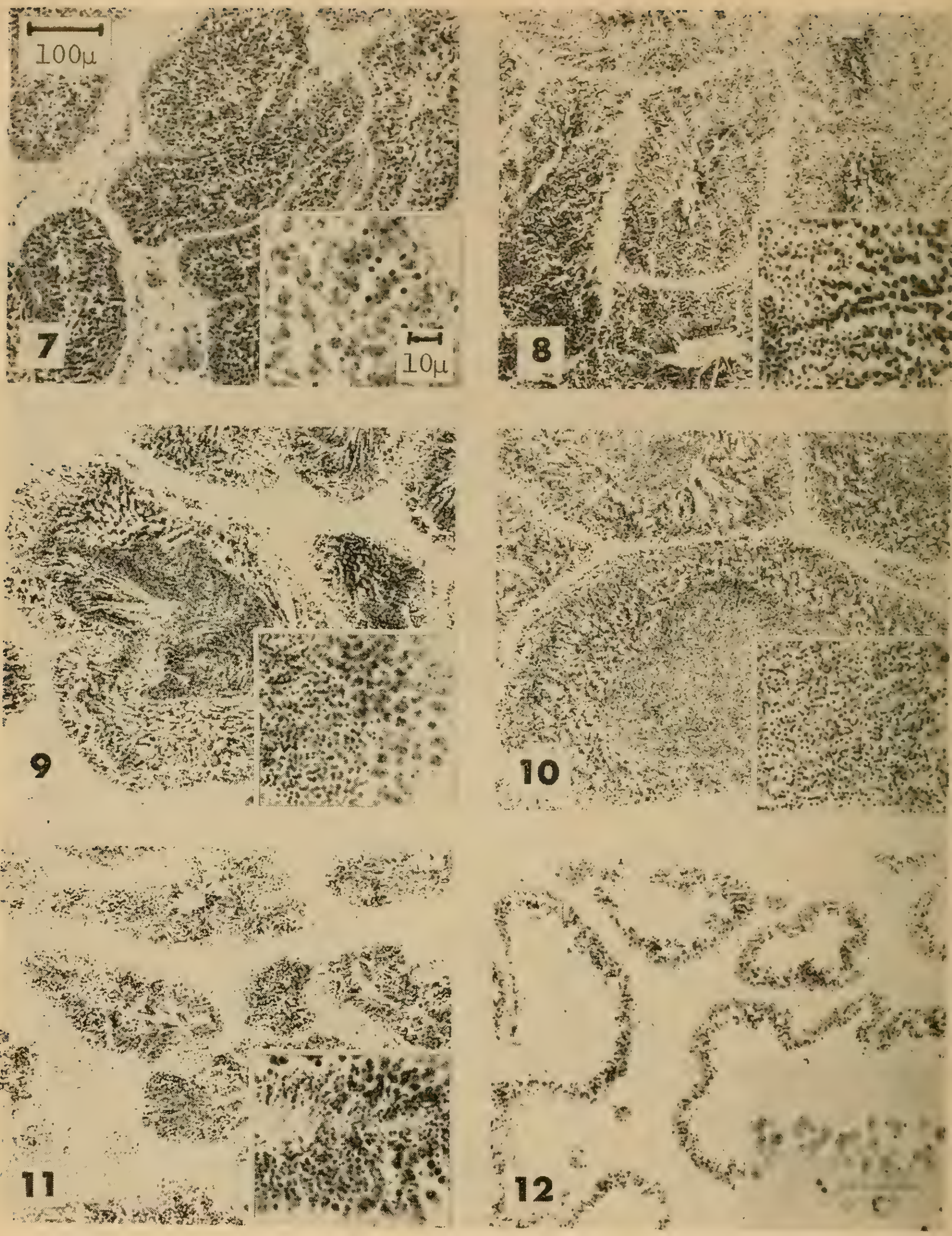
FIGURE 2. Same from a female surf clam, early active phase, showing enlarging ovocytes. Collection date: 18 February 1964.

FIGURE 3. Same, from a female surf clam, late active phase, showing large oval ovocytes. Collection date: 18 February 1964.

FIGURE 4. Same, from a female surf clam, ripe phase. Collection date: 26 June 1964.

FIGURE 5. Same, from a female surf clam, partially spent phase. Collection date: 28 August 1964.

FIGURE 6. Same, from a female surf clam, spent phase. Collection date: 13 August 1964.



FIGURES 7–12.

FIGURE 7. Section of gonad tissue from a male surf clam, early active phase of the first annual reproductive cycle. Collection date: 21 January 1964.

FIGURE 8. Same, from a male surf clam, early active phase, spermatozoa form columns. Collection date: 18 February 1964.

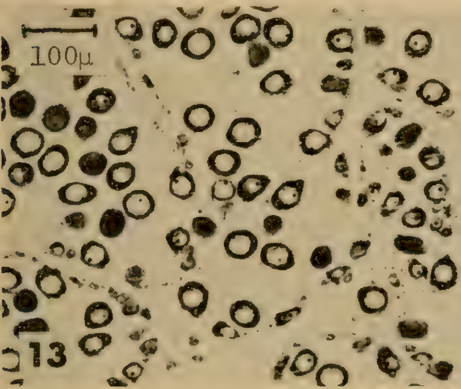
FIGURE 9. Same, from a male surf clam, late active phase. Collection date: 3 June 1964.

3. *Ripe phase*

Mature sperm form dense masses in the alveoli of clams in the ripe phase (Fig. 10). The sperm appear to be swirling in the centers of some alveoli, in others their tails are oriented toward the centers of alveoli, and in still others they form a homogeneous mass. Cells in early stages of spermatogenesis at the periphery of alveoli are much less numerous than the sperm.

4. *Partially spawned phase*

Partially spawned male clams still contain spermatozoa within the centers of alveoli, but they are substantially less numerous than in ripe clams (Fig. 11). The alveolar walls are compressed and thickened. Like the ovogonia and early ovocytes at this stage, spermatogonia and primary spermatocytes are developing along the basal membrane before most gonads are completely void of spermatozoa.



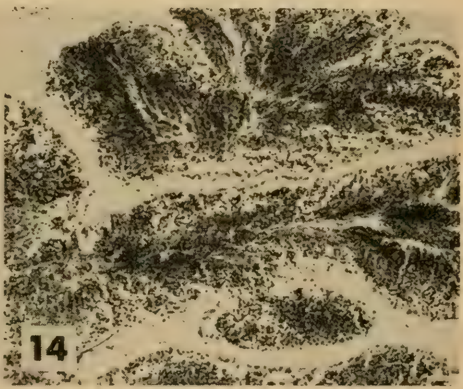


Figure 13. Section of gonad tissue from a female surf clam, ripe phase of the second annual reproductive cycle. Collection date: 24 September 1964.

Figure 14. Section of gonad tissue from a male surf clam, ripe phase of the second annual reproductive cycle. Collection date: 24 September 1964.

5. *Spent phase*

The alveoli of spent male clams contain no spermatozoa or very few and the lumina are characteristically open (Fig. 12). Primary spermatogonia are proliferating from the alveolar walls.

Second Annual Cycle

A second annual maturation of New Jersey surf clams was observed during three of the four years and was essentially a repetition of the sequence in gonad conditions already presented. The second maturation seemed to be of minor

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Figure 10. Same, from a male surf clam, ripe phase, a dense mass of spermatozoa is formed. Collection date: 15 July 1964.

Figure 11. Same, from a male surf clam, partially spawned phase. Collection date: 29 July 1964.

Figure 12. Same, from a male surf clam, spent phase. Collection date: 29 July 1964.

importance, however, since fewer ovocytes appeared in the gonadal alveoli (Fig. 13). Counts of the number of ovocytes in 1.25 mm.^{2} of gonadal tissue sections of ripe clams from the two reproductive cycles in 1964 showed an average density of 44.2 ovocytes for 32 ripe clams from the first cycle and 26.4 ovocytes for 20 ripe clams from the second cycle. The sperm in male gonadal tissues also appeared to be less numerous during the second cycle (Fig. 14).

Annual Reproductive Cycles in Relation to Water Temperature

An account of the gonad conditions in 1514 surf clams and bottom water temperatures taken in the area sampled for clams is presented in chronological order. Gonad collections were initiated in June, 1962, and continued through 1965; water temperature data were available for the period 1962–65. Unless otherwise specified, all references to water temperature are for the bottom.

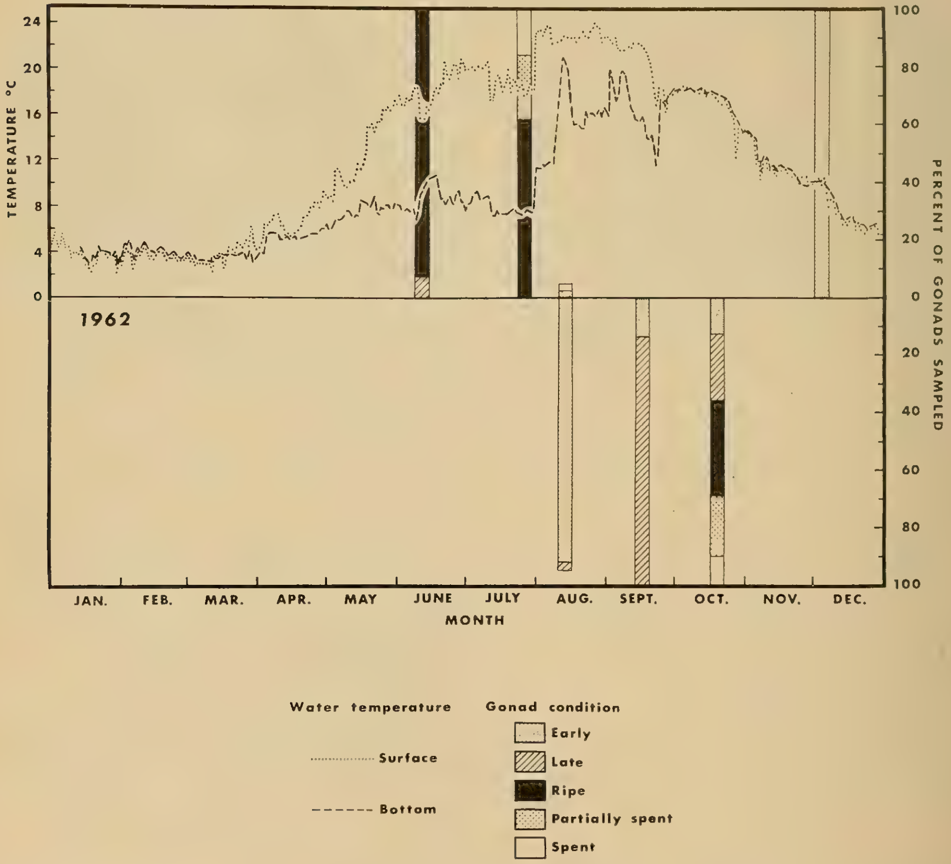

Figure 15. Gonad conditions of *Spisula solidissima* from New Jersey and sea-water temperatures at Barnegat Lightship in 1962. The bars above the centerline represent a single reproductive cycle and those below the centerline represent a subsequent cycle.

Reproductive cycle and water temperature in 1962

The June and July samples contained mostly ripe clams (Fig. 15). Spawning began by about mid-July when 37% of the clams were in the partially spawned and spent condition, and was completed by mid-August when 94% of the clams were in the early active phase. All clams were in the early and late active phases during September. All phases of gonad condition were represented in the October sample. A second spawning was apparent in mid-October when 32% of the clams were partially spawned and spent. All clams collected in early December were in the early active phase.

The temperatures were lowest (2.2° C.–5.2° C.) in late January, February, and early March. A warming trend followed, and 8° C. was reached by mid-May. The temperature reached 10.7° C. in mid-June, but most temperatures were between 7° C. and 9° C. during the 1½ months preceding the mid-July spawning. Spawning began 1 to 2 weeks before a rapid temperature rise, from 8° C. to 21° C., during late July to mid-August. Clams in the early active phase of a second reproductive cycle were most numerous when the temperature had reached its seasonal high (21° C.). The second spawning was in October, during a period when temperatures dropped from a high of 18° C. to about 10° C.

Reproductive cycle and water temperature in 1963

All of the clams were in the early active phase in January (Fig. 16). Gonads were in the late active phase in 4% of the clams by mid-February and in 56% of the clams by mid-March. Ripe clams constituted 92% of the mid-May sample and 98% of the mid-June sample. Spawning began in early July when 60% of the clams were partially spawned or spent. During August, 75% of the clams were still partially spawned or spent. The first annual spawning must have been completed by the end of August, because by mid-September 96% of the clams were in the late active and ripe phase. In mid-October collections, 28% of the clams were partially spawned and spent—evidence of a second spawning. Most (92%) of the clams were in the early active phase by early December.

Winter temperatures fell to a low of 2° C. during February. A warming trend after March (bottom temperatures are not available for March) was gradual and without marked fluctuations. The temperature on the bottom reached 10° C. by early June and 11° C. by late June. Temperatures reached 12° C. in mid-July. Spawning in July and August preceded a rapid rise in temperature from 9.6° C. to 18.9° C. during the early part of September. The beginning of a second reproductive cycle coincided with the rapid rise in temperature. The second spawning in October was during a period when temperatures dropped from 17° C. to about 15° C.

Reproductive cycle and water temperature in 1964

All clams were in the early active phase in January (Fig. 17); 63% were in the late active phase in February and this condition predominated in March and April. Gonads were ripe in 79% of the clams in May and in all of the clams in June. Spawning occurred in early July (43% of the clams were in the partially spawned and spent condition in mid-July). Fifty-three per cent of the clams were partially spawned and spent in mid-August and the rest were in the early active phase. A progressive development of the gonads during September and October was followed by a second spawning late in the year; 68% of the clams were partially spawned and spent in early November. In early December, 80% of the clams were in the early active phase.

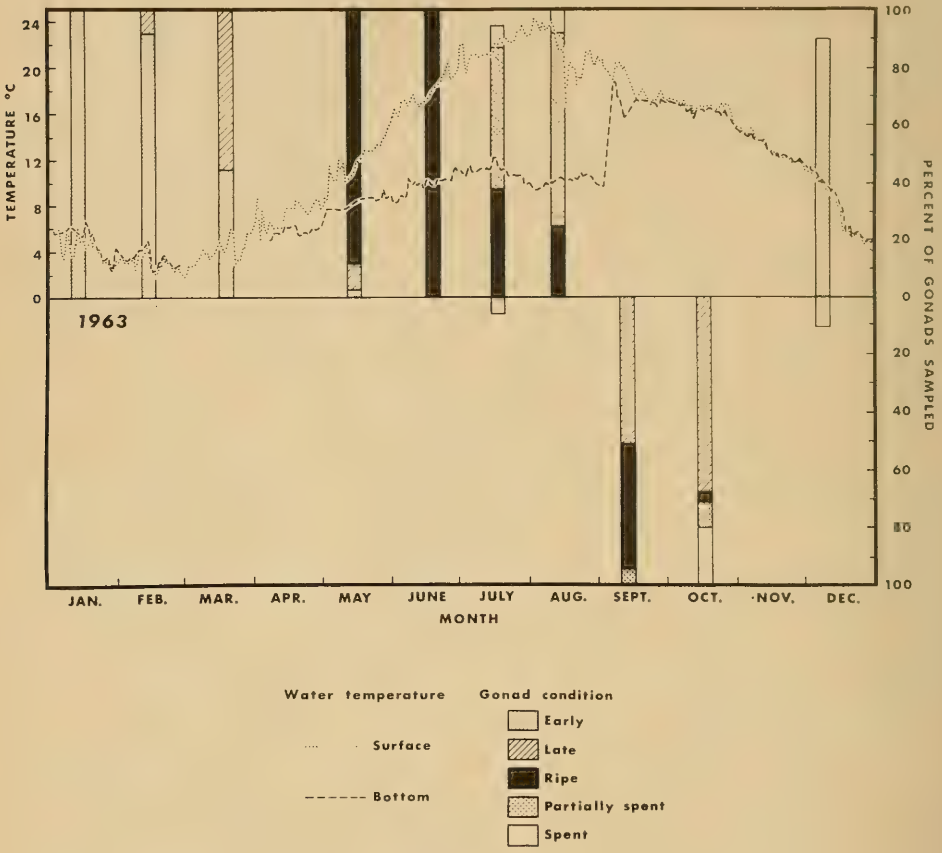

FIGURE 16. Gonad conditions of *Spisula solidissima* from New Jersey and sea-water temperatures at Barnegat Lightship in 1963.

The period of low winter temperature from January through March included a drop to 2° C. in February and was followed by a warming trend in April. In May and June the bottom temperatures fluctuated between 7° C. and 9° C. A temperature of 11° C. was reached in early July and was a possible inducement to spawning. All of the clams were completely spawned out in August after the rapid rise in temperature (7.4° C.–18.6° C.) during late July. The second reproductive cycle was underway in 47% of the clams after the rapid rise in temperature. The second spawning was during a period when temperature dropped from 16° C. to about 12° C.

Reproductive cycle and water temperature in 1965

All clams were in the early active phase during January and February (Fig. 18). The late active phase appeared in 76% and 62% of the clams during March and April, respectively. Ripe clams made up 94% of the late June sample and continued to predominate in the July, August, and September samples. In July and August only 7% to 11% of the clams were partially spawned and from mid-September to mid-October 29% to 52% of the clams were partially spawned or spent. Thus a mid-year spawning observed during the 3 previous years of the study was delayed 2 months in 1965. A few clams (7% to 13%) were in the spawning condition in late November and mid-December. From mid-October through December, however, many clams (48% to 93%) were in the early active phase. No second reproductive cycle was observed.

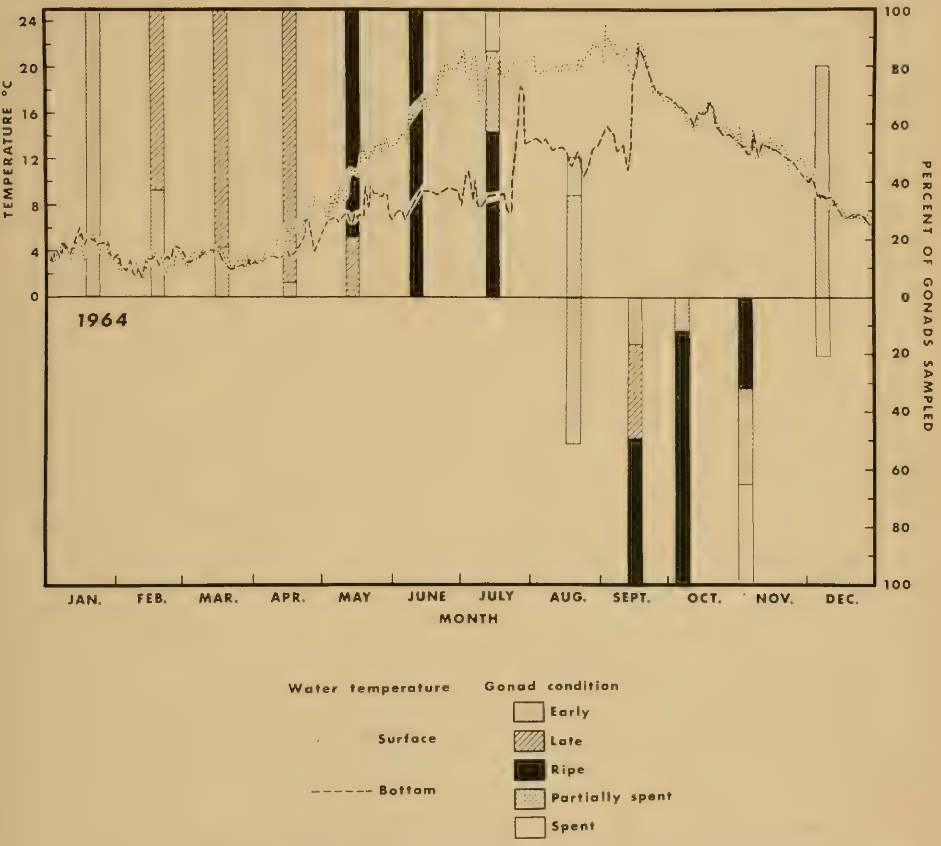

FIGURE 17. Gonad conditions of *Spisula solidissima* from New Jersey and sea-water temperatures at Barnegat Lightship in 1964.

Low winter temperatures were approximated from the surface observations because bottom temperatures were not available until early May. Warming to about 6° C. in May was followed by temperatures of about 8° C. in June and July; fluctuations of 2° C. occurred but the temperature did not exceed 10° C. Temperatures of 11° C. were recorded on three occasions in August. Temperature rose sharply from 10° C. to 18° C. in mid-September. Unlike the sequence in the 3 earlier years, spawning apparently occurred after the sharp rise in temperature.

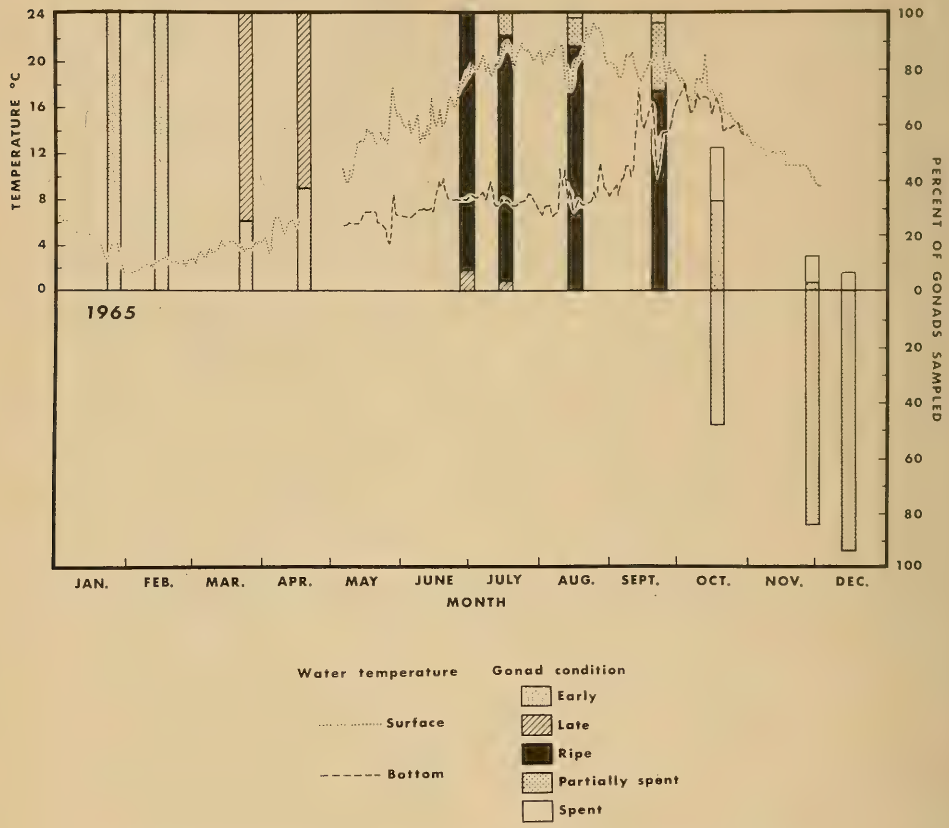

FIGURE 18. Gonad conditions of *Spisula solidissima* from New Jersey and sea-water temperatures at Barnegat Lightship in 1965.

DISCUSSION

In this study the term "reproductive cycle" includes the earliest detection of sex cells during periodic histological examinations of gonad tissue, the growth of female sex cells and multiplication of male sex cells which eventually fill the gonadal tubules, and the disappearance of sex cells from the tubules during the spawning period. The reproductive cycle can be separated into the gametogenic period (*i.e.*, the early, late, and ripe phases) and the spawning period (*i.e.*, partially spawned and spent phases). No period of inactivity was apparent in the sections of surf clam gonads, since sex cells for a succeeding reproductive cycle were detected in spent individuals (Figs. 6 and 12). In fact, separating the reproductive cycles of some marine invertebrates into an "inactive phase," "rest period," "quiescent period," or "vegetative phase" may only be a convenience (Ropes and Stickney, 1965). Biological processes maintaining life and early gametogenesis could very well be taking place during the inactive phase and be undetected by the histological technique used.

Examinations of the slide preparations of gonads provide an accurate basis for describing the reproductive cycle of surf clams from offshore New Jersey. Sex cell development began during December and, concurrent with the enlargement of the gonads, was followed by the occurrence of numerous partially spawned and spent clams during July and August each year from 1962 through 1964. A second maturation of sex cells followed the mid-year spawning, but gonad distention was minimal. Partially spawned and spent clams in the mid-October to early November samples in 1962 through 1964 was evidence of a second annual spawning. Although a biannual reproductive cycle may be typical for surf clams from offshore New Jersey during most years, a single reproductive cycle and spawning in 1965 was an important exception. The time and frequency of spawning each year, then, can vary.

Detection of a biannual reproductive cycle in surf clams from offshore New Jersey would be difficult without microscopic examinations of gonads throughout several calendar years. Westman and Bidwell (1946, see footnote 1) reported a mid-year spawning of surf clams from offshore Long Island, New York, by observing gonad distention and shrinking after spawning, but their method of visually determining gonad condition might not be precise enough to identify a second annual spawning, if one occurred. Allen (1951, 1953) observed a scarcity of eggs in surf clams from Woods Hole, Massachusetts, during 1 to 2 weeks in mid-summer coincident with a period of excessive heat. A possible explanation is that the clams had spawned or, since he continued to collect eggs for a 2- to 3-month period after mid-summer, these eggs may have been produced during a second annual reproductive cycle.

Temperature frequently influences the reproductive cycle and spawning of marine invertebrates (Giese, 1959; Loosanoff and Davis, 1963). During the gametogenic period, the surf clams in the beds off New Jersey were surrounded by gradually increasing, but fairly stable temperatures for the first six months in the three early years of the study. A progressive development of the gonads to a turgid and ripe state during the period of warming to temperatures of 12° C. or a little less preceded the first annual spawning. In 1965, however, the water temperatures during the gametogenic period were generally colder (8° C. or less) and more erratic (highs to 11° C.) than in the previous years. Gametogenesis was slightly delayed by the lower temperatures—94% of the clams were in the ripe phase by late June, 1965, whereas 100% were in the ripe phase by mid-June of 1962, 1963, and 1964.

Temperature was not clearly a stimulus to spawning of surf clams in their natural beds off New Jersey. Spawning by some clams did not accompany abrupt temperature changes in all 4 years, although more clams spawned before the abrupt temperature change in 1962–64 than in 1965. Spawning by some bivalves can be effectively delayed by holding them at low temperatures. Conversely, gonad development to a ripe condition has been hastened by holding bivalves in warm sea water.

The ripe bivalves have been spawned "out of season" and have produced normal larvae (Loosanoff and Davis, 1963).

I have held surf clams in 20° C. water from late February to mid-April, 1967 and compared their gonadal development with that of clams from the parent population in Chincoteague Inlet, Virginia. Gonads of all clams in the inlet were in the late active phase in late February and remained in this condition until after April. Water temperatures during the experimental period gradually increased from 5° C. to 11° C. In contrast, 10 gonads from clams held in the warmed water were all in the ripe phase by mid-March. On 11 April, a female spawned spontaneously in one of the containers and 2 partially spawned individuals were found when gonad sections of 10 clams were examined. These three specimens, then, spawned without the stimulus of a thermal shock and were perhaps like the ripe individuals from the beds off New Jersey that spawned before an abrupt temperature change.

The surf clam apparently develops its gonads to a ripe condition within a short period of time, contains ripe sex cells for several months of the year, and responds readily to relatively simple spawning stimuli. A rapid gonad development may account for the report by Carson (1945) that the surf clams of the mid-Atlantic coast spawn in the spring and throughout the summer. Allen (1951, 1953) obtained eggs of New Jersey coast clams from early spring to late autumn. Schechter (1941) collected ripe surf clams from areas near Woods Hole, Massachusetts, throughout the summer. Loosanoff and Davis (1963) found morphologically ripe eggs and spermatozoa in surf clam gonads from mid-January to the end of June. Conditioning the clams in the early months of the year by placing them in warmed water produced ripe specimens. Later in the season, when the natural waters had reached 12° C., no conditioning was needed, and thermal stimulation alone was sufficient to induce spawning. In contrast, other species of bivalves in the laboratory require various physical and chemical stimuli such as electrical shocks, rhythmic changes in water temperature, the addition of ammonium hydroxide to the water or injected into the gonad, or an introduction of egg or sperm suspensions to the water to induce spawning—and even then some species do not respond (Galtsoff, 1961, 1964; Loosanoff and Davis, 1963; Stickney, 1964).

The importance of nervous and hormonal interrelationships in bivalves has been reviewed by Galtsoff (1961, 1964) and Giese (1959). Products of neurosecretory cells inhibit spawning, and bivalves become sensitive to environmental spawning stimuli after the cells disappear. Degeneration of the neurosecretory cells begins a few days before spawning. Nagabhushanam (1963a) recognized two types of neurosecretory cells in the cerebral and visceral ganglia of *Spisula solidissima*. The cells in surf clams appear morphologically similar to those in the oyster, *Crassostrea virginica*, and the mussel, *Modiolus demissus* (Nagabhushanam 1963b, 1963c, 1964). Experiments with the latter two species link the cells with the hormonal control of the gametogenic cycle and spawning. Stimuli such as low salinities, electrical shocks, and high temperature influenced the secretions.

Both Galtsoff (1961) and Stickney (1964) believed that ripeness, rather than specific temperature, influences the response of some bivalves to spawning stimuli. Ripeness and sensitivity to spawning stimuli seem to precede the natural spawning period of surf clams by many months. I classified surf clams from New Jersey as ripe for at least two months each year before the first annual spawning. Some were induced to spawn in late May and early June, 1965, by simply placing them in running sea water aboard a research vessel. At four locations where the clams were caught, the water temperature increased as little as 9.5° C. and as much as 11.5° C. from bottom to surface. Changes in hydrostatic pressure or shocks from being caught by the jet dredge might stimulate spawning, but none of the clams from a fifth location spawned when the temperature change was only 0.8° C. A response to the stimuli of an abrupt change in temperature, well in advance of the seasonal time of spawning, suggests that the hormonal control of spawning can be overridden in surf clams. Nevertheless, a minimum temperature threshold for a spawning response is probably necessary.

Sections of surf clam gonads from geographic locations other than the commercial fishing areas off the New Jersey coast were examined, since a biannual reproductive cycle and spawning may not be typical throughout their range. In monthly samples from a more northern habitat—Sagadahoc Bay, Maine—during May through September, 1962, only a single reproductive cycle was apparent. Partially spawned and spent clams in the August and September samples were evidence of a late summer and early fall spawning.

In other bivalves, the reproductive cycle changes with latitude. Pfitzenmeyer (1962, 1965) and Shaw (1962, 1965) observed a biannual reproductive cycle in soft-shell clams, *Mya arenaria,* from Chesapeake Bay, Maryland, the southern part of their range. Ropes and Stickney (1965) reported on a single annual cycle in soft-shell clams from areas north of Cape Cod, Massachusetts, and discussed the available evidence of a biannual cycle and spawning south of Cape Cod. Porter (1964), who described the seasonal gonadal changes in hard clams, *Mercenaria mercenaria,* from North Carolina, found two annual spawning periods. Both of these species apparently have only one reproductive cycle each year, but two in southern waters. The change in the breeding season with respect to latitude and an extension of the season as one goes south has been observed in many temperate marine invertebrates (Giese, 1959).

I wish to gratefully acknowledge the cooperation of the members of the surf clam industry and many of the commercial fishermen who welcomed me aboard their vessels to sample clams from their catches at sea. The technical assistance of Virginia A. Liddell, Sally V. Otto, Brenda Spriggs, and Geraldine J. Fitzgerald aided me in the preparation of the many slides for the study. Dr. Rudolf S. Scheltema, Woods Hole Oceanographic Institution, Woods Hole, Mass., reviewed the manuscript and made many helpful suggestions. Dean F. Bumpus, Woods Hole Oceanographic Institution, Woods Hole, Mass., furnished the unpublished temperature records at Barnegat Lightship for 1964 and 1965.

SUMMARY

1. A biannual reproductive cycle was observed in *Spisula solidissima* gonads during 3 of the 4 years in which samples were taken from offshore New Jersey.

2. The biannual cycle was characterized by a major mid-year spawning and minor late-year spawning. A second annual reproductive cycle may be neither typical for surf clams throughout their geographical range nor always an annual event in clams from New Jersey.

3. A delayed spawning and single annual cycle in 1965 was related to colder environmental temperatures than were observed in the three earlier years of the study.

4. Abrupt temperature changes were not clearly a cause of spawning in the natural populations of surf clams. Partially spawned clams were found in the samples in advance of abrupt temperature changes. Temperature, however, clearly delays or hastens the gametogenic cycle and stimulates spawning of ripe surf clams in the laboratory.

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THE GROWTH AND SIZE DISTRIBUTION OF CURRENT YEAR CLASS LOLIGO PEALEI[1]

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Loligo pealei (Lesueur, 1821) is the common squid between Cape Cod and Cape Hatteras (Verrill, 1882). It is abundant inshore during the warmer part of the year and is concentrated near the Continental Shelf break in the winter time (Summers, 1967). In spite of its ubiquity, its apparent importance in pelagic food chains and its significance as a source of "squid giant axons," the life history of *L. pealei* has not been fully described.

Verrill (1882) and Haefner (1964) both estimated the growth rate of this species by equating different year classes with size-frequency modes. By this method, Verrill suggested that the largest individuals were three or four years old and Haefner demonstrated two distinct year classes. Their analyses were subjective when the size classes overlapped each other and contained the assumption that *L. pealei* grows at a continuous, if not uniform, rate through its life span.

Verrill's report was based upon a synthesis of preserved specimens and was presented as a description of the growth rate of *L. pealei* off the "southern coast of New England." His tabulated data included measurements of approximately 3378 individuals, more than 2600 of which were collected in Vineyard Sound, south of the Cape Cod peninsula. Haefner cited morphometric data on 1165 individuals taken in Delaware Bay during the summer of 1958. His size-frequency information was pooled over the collecting period and consequently cannot be used to demonstrate short-term growth. Neither study fully accounted for the sampling bias of the collecting gear.

The abundance of *L. pealei* in Vineyard Sound during the warmer part of the year is known from Verrill's collections and from the work of Sumner, Osburn and Cole (1913). The latter dredged specimens throughout the waters surrounding Woods Hole, Massachusetts, and indicated a concentration in the southwestern part of Vineyard Sound. During the past five summers, the fishing vessel CAP'N BILL IV, under charter to the Marine Biological Laboratory, has trawled for this squid on 181 different occasions in the southwestern part of Vineyard Sound.

The present paper attempts to more precisely describe the early growth and size distribution of *L. pealei* in Vineyard Sound. The study was facilitated by dealing primarily with the numerous, rapidly growing current year class (individuals less than one year old) and by the fact that Vineyard Sound is near the northern range limit of this species where the breeding season is relatively

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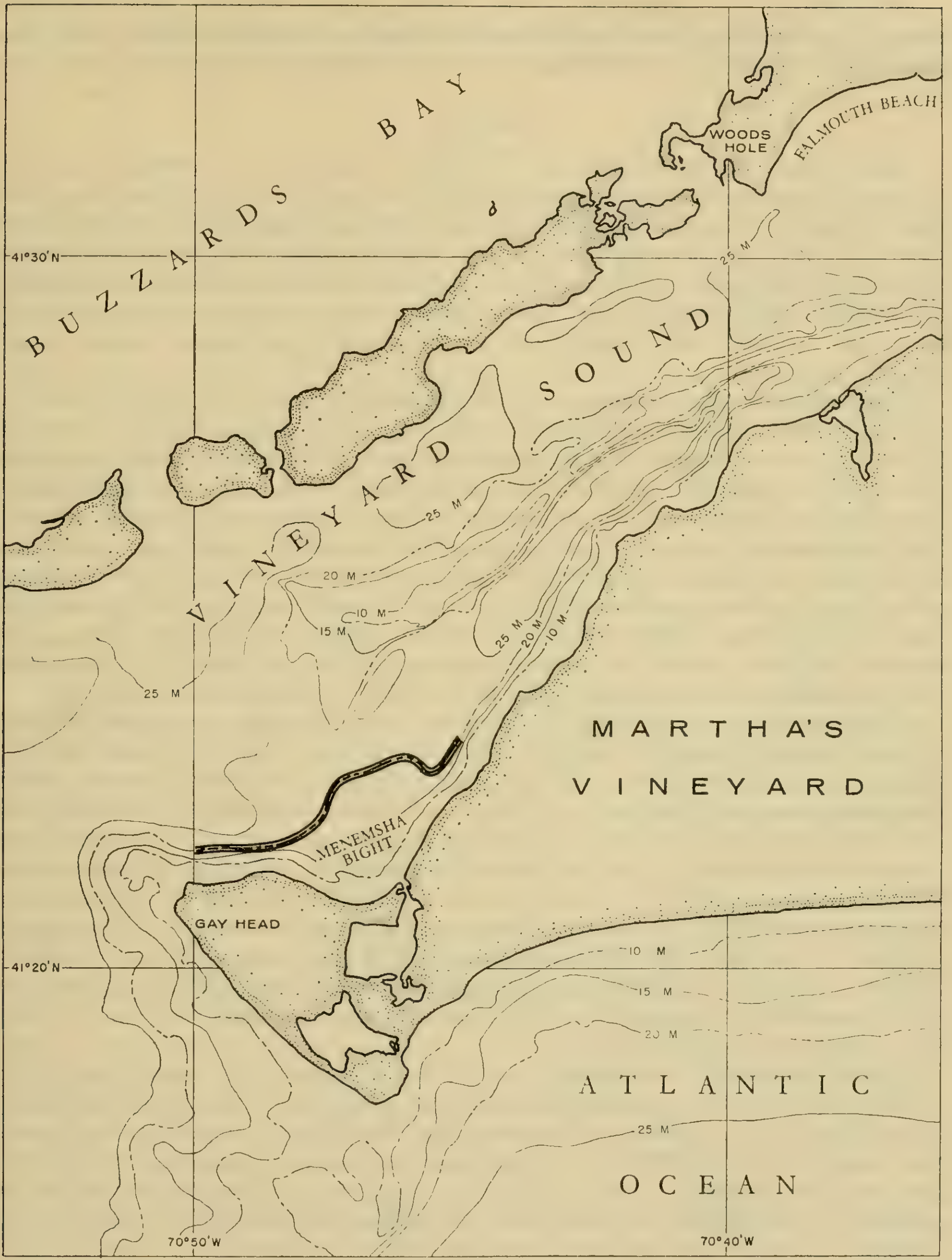
BUZZARDS BAY
WOODS HOLE
FALMOUTH BEACH
41°30′N
25 M
VINEYARD SOUND
25 M
20 M
10 M
15 M
25 M
25 M
20 M
10 M
MARTHA'S VINEYARD
MENEMSHA BIGHT
GAY HEAD
41°20′N
10 M
15 M
20 M
ATLANTIC OCEAN
25 M
70°50′W
70°40′W

FIGURE 1. A contour map of Vineyard Sound. Menemsha Bight is located at 41° 22′ N, 70° 48′ W. The mean water depths are given in meters; the sampling region is shown by a heavy 20-m. contour line in Menemsha Bight.

short. Sampling was restricted to one location and procedures were consistent throughout the study in order to determine seasonal changes. This work was undertaken as a part of a continuing study of the ecology of *L. pealei.*

METHODS

The growth rate and longevity of *L. pealei* could not be determined in the laboratory because this animal can not be reared in captivity. A program of tagging and recapture of squid in the field did not seem practical short of a large scale operation. The remaining option was to repeatedly determine the size-frequency of a natural population and infer growth from the changes between sampling dates. This method was employed knowing that it introduced the least experimental bias in growth estimates and required the greatest care in sampling from a single population.

The site chosen for this study was Menemsha Bight (41° 22′ N, 70° 48′ W), a shallow open embayment at the southern extreme of Vineyard Sound, west of Martha's Vineyard Island. As illustrated in Figure 1, Menemsha Bight is partially protected from the open waters of the Atlantic Ocean by the Gay Head promontory. The Bight has an unobstructed, gently sloping sandy bottom and normally does not have tidal currents in excess of 3 km./hr. There is little temperature stratification in Vineyard Sound due to significant water movement and the annual surface temperature cycle ranges from 0 to 22° C. in February and August, respectively. Sea-water salinities remain consistently at 31 to 32‰ through the year. The sampling region is shown by a heavy 20-m. contour line in Figure 1. This region was accessible for otter trawling for a linear distance of approximately 5 km.

Squid were taken with a #35 otter trawl towed by the stern rigged, 65-foot (20-m.) research vessel A. E. VERRILL (named for the author cited above). Except for the addition of a 1.25-inch mesh (3.2-cm.) stretched measure nylon liner in the cod end, this was a typical New England commercial groundfish net. The trawl doors weighed 500 lb. (225 kg.) apiece and each was separated from the net by 12 fathoms (22 m.) of groundlines and legs. The headrope and footrope measured 52 and 72 feet (16 and 22 m.), respectively and the forward part of the net was made of 42-thread nylon twine with 5-inch mesh (13-cm.) stretched measure in the wing and square sections and 4½-inch mesh (11-cm.) stretched measure in the belly and cod end. The headrope had 12 8-inch (20-cm.) aluminum floats spaced along its length and the foot rope was protected by 4- and 6-inch (10- and 15-cm.) rubber rollers.

All of the samples were taken near midday (earliest 1031 hours, latest 1422 hours) and the tows averaged 50 minutes in duration. The towing speed was 6.5 km./hr. except on the first two sampling dates when a slightly slower speed was employed. The contents of the net were dumped into large tubs on the deck and sorted immediately. The entire squid catch, or a representative sample of several hundred individuals, was measured and sexed as soon as possible after capture. No preserved specimens were included in the results.

The dorsal mantle length was recorded for every individual in the sample; squid less than 5 cm. were measured to the nearest whole millimeter, larger squid were measured to the nearest whole centimeter. The ventral mantle wall was slit open and the sex was recorded for all but the smallest, immature individuals. Males were considered mature when spermatophores were present; this was always accompanied by a thickening and white coloration of the vas deferens. Females were considered mature when the ovary was expanded and loose eggs were found in the oviduct.

RESULTS

A total of 1619 individuals were measured from samples collected in Menemsha Bight on seven consecutive dates ranging from August 18 to November 16, 1967. The numbers of squid caught on November 2, 16 and 22 were approximately 1000, 5 and 0, respectively, and none were taken through March of the following year. *L. pealei* was the only species of cephalopod taken. Many species of fish were caught, some in abundances of several hundred pounds per tow.

It is apparent from the size-frequency data for squid taken on the first sampling dates that current year class *L. pealei* were represented by a few small individuals mixed in with the larger size classes. This class grew and became more dispersed in later samples and eventually merged with the larger classes in late September. The current year class was initially characterized by a narrow size range and illustrated a marked skewness toward the larger sizes. Examination of the various size-frequencies showed that the current year class and the older classes were adequately approximated by lognormal size distributions.[2] Thus, it was possible to more rigorously separate the size classes on lognormal probability paper using the method described by Cassie (1954, 1962). Appropriate age categories could be assigned to these size classes by contrasting the collections on different dates.

The best fit size distributions for six sampling dates are shown in a lognormal probability presentation in Figure 2. Age classes are indicated by brackets to the right of the best fit lines in this figure. The amount of class overlap can be seen in Figure 2 by the horizontal coincidence of different age classes on a single sampling date. It should be noted that Cassie's method of size class separation is based upon size distributions and is little affected by exceptional individuals or the location of the modal size. The latter generally occur at a point below the cumulative 50% in Figure 2.

The current year class made up an increasing proportion of the samples during the time covered by this study. The percentage of current year class individuals in the measured samples is shown for various dates in Figure 3. The dashed line refers to a sizeable catch of *L. pealei* (308 individuals) taken with a 16-foot (5-m.) shrimp trawl under the same conditions give above. There were no current year class squid in this sample and the one plus year class had 5th, 50th and 95th percentile lognormal fit mantle lengths (equivalent to the ends and middle of the lines shown in Figure 2) of 6.8, 9.3 and 12.7 cm., respectively. The dotted line on November 16 indicates the last date of squid capture for the 1967 season.

Figure 4 shows a growth scheme for the current year class *L. pealei* based upon the best fit lognormal size distributions illustrated in Figure 2. The bars

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[2] A lognormal size distribution is one in which the logarithm of the variate (in this case, mantle length) is normally distributed. Size distributions in this report are all positively skewed, implying that the modal size lies closer to the lower range size than the upper range size. A positively skewed lognormal distribution restricts the variate to values greater than zero.

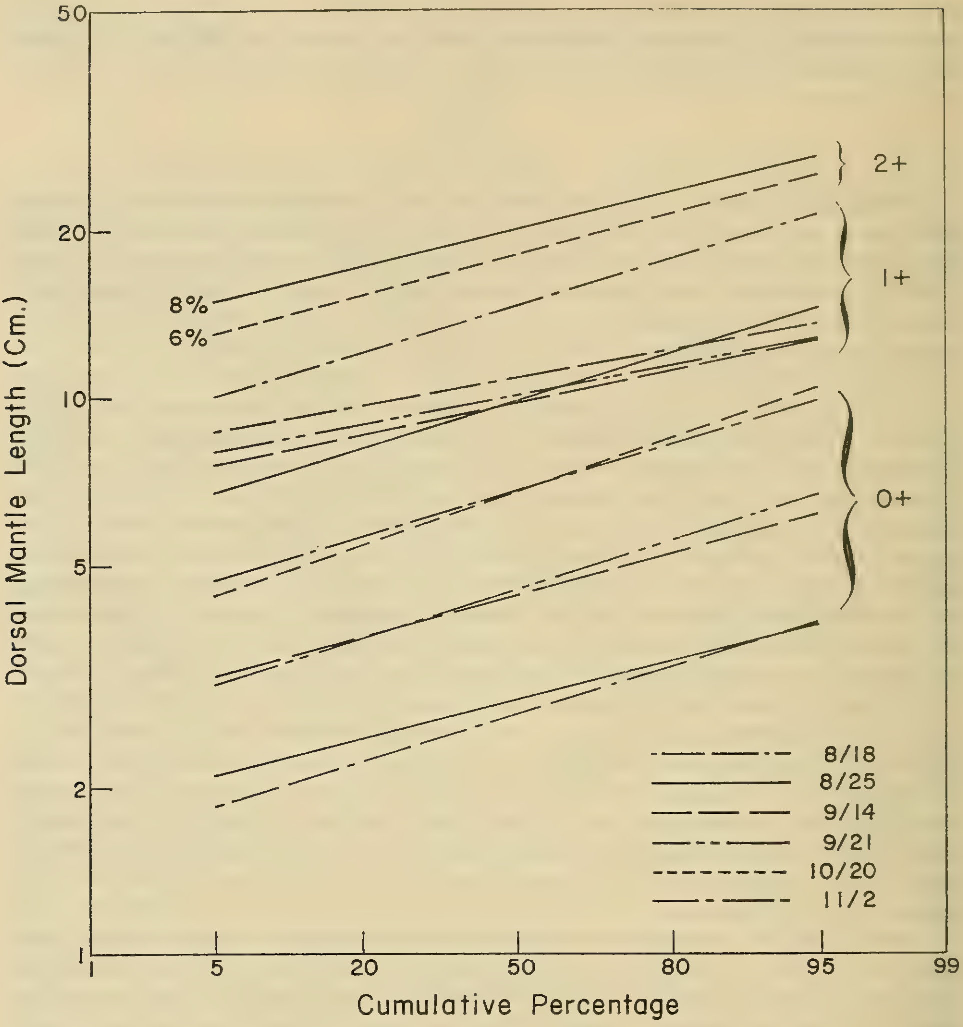

FIGURE 2. Best fit lognormal size-frequencies for different age classes of *L. pealei* resulting from six collections. This is a lognormal probability presentation of the data. Age classes in years are shown by brackets to the right of the fit lines. Sampling dates are indicated by the line codes given in the figure legend. The percentage occurrence of two plus year class individuals in the samples is shown by numerals to the left of the fit lines.

extend from the 5th to the 95th percentile sizes and the dots represent the median, or 50th percentile size. Linear regression lines are given each of these percentages and drawn out from the hatching size (2 mm. mantle length) to the date of last squid capture, November 16. The stippled area in this figure represents Verrill's conclusion on the growth rate of *L. pealei* off the "southern coast of New England." These reports do not agree in date, growth rate nor size distribution.

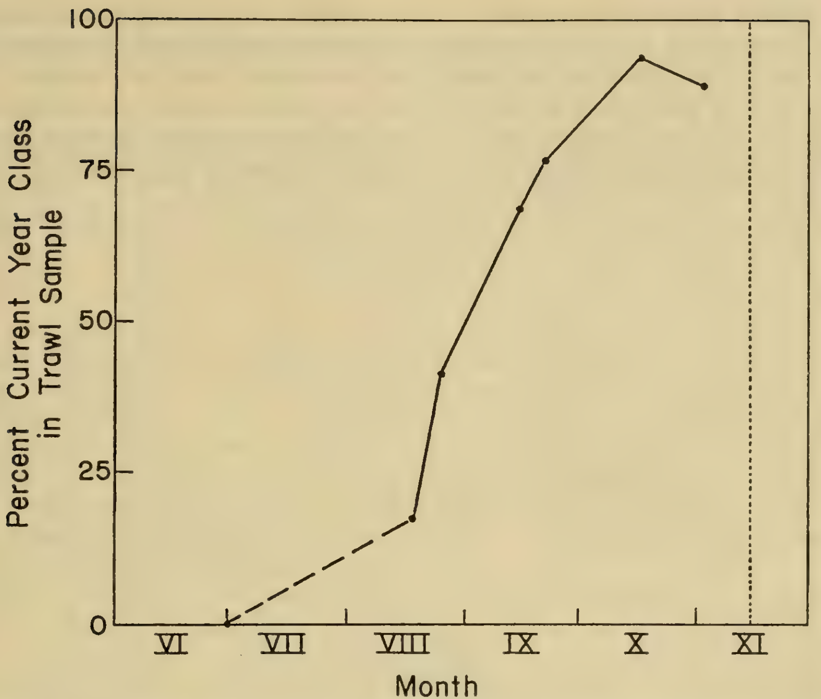

FIGURE 3. Percentage of current year class individuals in trawl samples for various dates. The dotted line on November 16 indicates the last date that *L. pealei* was captured in Menemsha Bight before its fall migration. A dashed line connects with data from a collection made with a shrimp trawl.

DISCUSSION

The accuracy of the foregoing estimates of growth rate and size distribution is dependent upon how well the measured samples represent the natural squid population. Owing to the mobility and seasonal migration of *L. pealei,* one can readily question the adequacy of daytime, otter trawl samples from a single depth zone and location. Furthermore, the size bias introduced through the collecting gear needs to be established in interpreting the results.

The consistency of the squid population in Menemsha Bight can only be implied by the regularity of sample size-frequencies and by comparison with other sources. The latter produced no significant differences when applied to records of miscellaneous collections on comparable dates in 1967 from Buzzards Bay, Vineyard Sound and Nantucket Sound, resulting from otter trawling, fishtrap hauls, squid jigging and fish stomach contents. Winter offshore collections of *L. pealei* show an increase in the numbers of individuals trawled in daylight hours and a positive correlation between depth and mean mantle length. This last factor may explain the size constancy of the one plus year class shown in Figure 2, by suggesting that this class may have been migrating through the sampling region during the study.

L. pealei does migrate vertically. For instance, newly hatched fry congregate near the surface in the best lit part of an aquarium and, during the warmer part of the year, adult squid are frequently seen swimming near the surface at night. The #35 otter trawl fishes in the first one to two meters above the bottom and it may well miss squid in upper strata. I suspect that the largest squid either swim high enough above the bottom to be missed by this net or are capable of avoiding

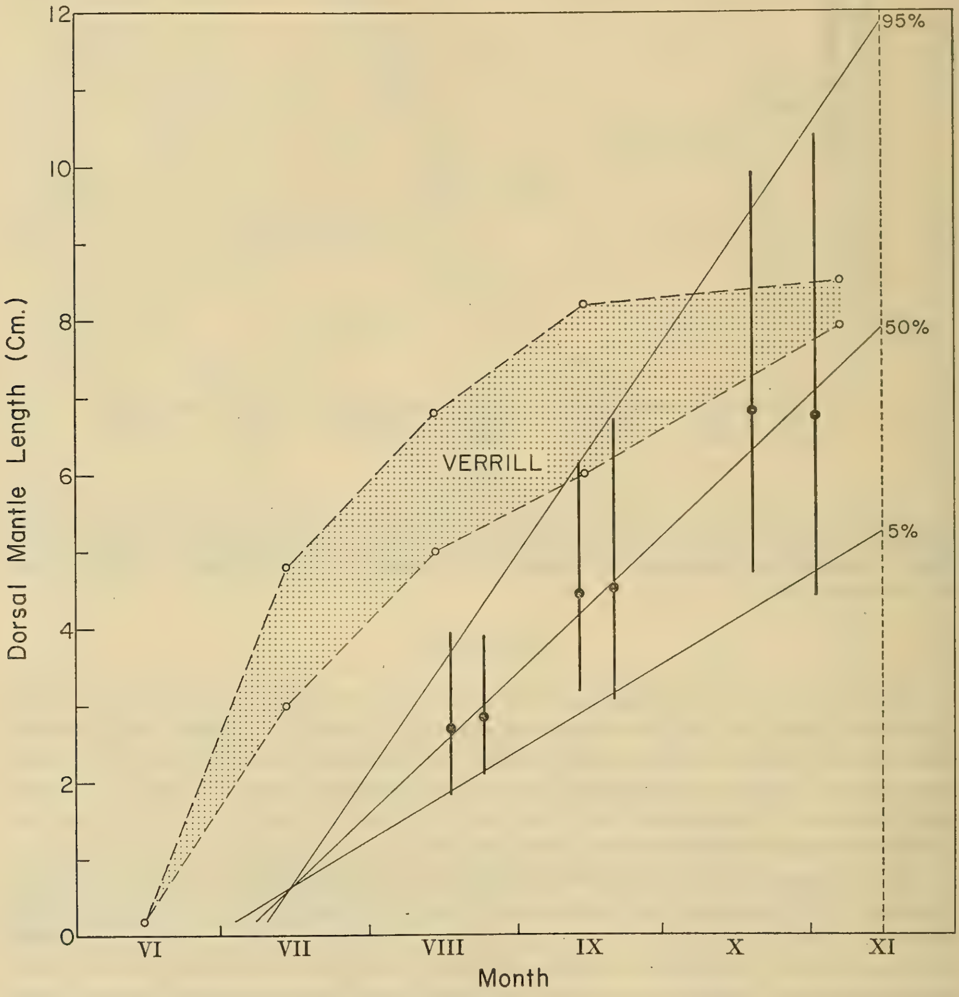

FIGURE 4. Two growth schemes for current year class *L. pealei.* The dorsal mantle length in centimeters is shown as a function of time. Verrill's data (1882) are indicated by a stippled envelope including the typical size range of his collections. The present study is represented by bars and dots showing the 5th, 50th and 95th percentile lognormal fit mantle lengths on six sampling dates. Linear regression lines are given for each of these percentile sizes. The dotted line on November 16 indicates the last date that *L. pealei* was captured in Menemsha Bight before the fall migration.

it, as the biggest individuals I have measured (including one male with a dorsal mantle length of 46.5 cm.) were taken in a fishtrap.

Selectivity

The selectivity of an otter trawl refers to the fact that it may retain individuals of different sizes with different efficiencies. Obviously, small individuals can slip through the net meshes and larger ones will be retained. Thus, selectivity is specified by the size *vs.* retention characteristics of a particular mesh size and species. This matter is discussed for several species of fish by Clark (1963).

Otter trawl selectivity can be estimated by comparing the catches of netting with different mesh sizes either on the same trawl or on similar nets fishing under comparable conditions. Such a comparison was made between the current year class *L. pealei* taken with the #35 net in Menemsha Bight on August 18 and two collections made by the Bureau of Commercial Fisheries R/V ALBATROSS IV which trawled in the same area at midday on August 17 and 18. The ALBATROSS IV used a #36 otter trawl with a $\frac{1}{2}$-inch mesh (1.5-cm.) stretched measure nylon liner in both the cod end and upper belly; it was 4 feet (1.2 m.) longer in each wing than the #35 net and more heavily protected for fishing on rough ground, but otherwise the nets were identical.

There were a total of 319 current year class individuals in these three collections, only 21 of which were caught in the #35 net. A comparison did not show a size bias in the #35 net sample and suggested that the entire size class straddled the transitional retention zones of the two trawl liners which had caused the #35 net to be uniformly less efficient in retaining this collection of squid. The 5th, 50th and 95th percentile lognormal fit mantle lengths for the ALBATROSS IV collections of August 17 and 18 and the A. E. VERRILL collection of August 18 were: 1.7, 2.2, 2.9; 1.9, 2.6, 3.4 and 1.9, 2.7, 4.0 cm., respectively.

The selectivity of the #35 net is probably the single most important factor in the explanation of the sample size class composition illustrated in Figure 3. As the current year class *L. pealei* grew, it was more efficiently retained by the net, and made up a larger proportion of the total catch. By assuming that the current year class made up 100% of the natural squid population and replotting Figure 3 on the basis of mantle length *vs.* per cent current year class in the samples, the 50% retention point for the #35 net was estimated to occur at a mantle length of 3.7 cm. This is consistent with the previous comparison and with Clark's data for fish.

Size distribution

Lognormal size-frequency distributions are not uncommon in juvenile animals and tend to occur in older animals where several age classes overlap in size and where mortality has reduced the abundance of the older classes. The mathematical philosophy of this distribution is contained in a monograph by Aitchison and Brown (1957); I will suggest some of the factors which may relate to the present case.

Net selectivity can cause the truncation of a size-frequency distribution if the smaller individuals are retained less efficiently than the larger ones. The A. E.

VERRILL collection of August 18 lay almost entirely below the 3.7 cm., 50% retention point, of the #35 net, and could be lognormally distributed as a result of net selectivity. Theoretically, the #36 net collections of the same and the previous day should have been influenced by a 50% retention point smaller than 3.7 cm. by the ratio of the liner mesh sizes (approximately 1.0 cm. mantle length when the twine diameters are taken into consideration). For practical purposes, the #36 net should not have shown a selectivity bias in the current year class collections of those dates. The size-frequencies of the ALBATROSS IV collections were strongly lognormal, indicating the condition of the natural population. This is borne out by the maintenance of lognormality in later collections and in older year classes.

A sexual dimorphism in the size-frequency relationship accompanies the onset of maturity in *L. pealei.* Haefner placed the inception of this dimorphism at a mantle length of 9.5 cm. and Verrill concluded that it took place at an age of eight months or a size of 6.2 to 18.8 cm. Thus, each of the older year classes shown in Figure 2 is a sum of two overlapping sex classes; the mature males generally larger than the females. The introduction of additional classes may help to explain the lognormality of the summed older year classes taken in contrast to the current year class. It was this contrast which made Cassie's method useful in the present study.

The relative abundance of the sexes did influence the size-frequencies of the samples. There were almost three times as many females as males in the one plus year class taken with a small trawl on June 30. This ratio was reduced to 2:1 on August 18 and remained approximately 1:1 thereafter. The two plus year class was composed mostly of mature males on August 25. Non-current year classes on October 20 and November 2 were poorly represented and scattered in size; as a result, their placement as two plus and one plus year classes, respectively, is tentative and based upon maturities. The current year class remained sexually immature throughout the sampling period, as did approximately one-half of the one plus year class. Spent females were rare in the collections.

Growth rates

The upper size limit in Verrill's growth scheme, as illustrated in Figure 4, was apparently constructed from the interface between mature and immature individuals listed in his tabulated results. It was his assumption that *L. pealei* matures in one year, an assumption that requires an individual to be a mature participant in 3 to 4 breeding migrations if it lives to that age. The size class separation shown in Figure 2 suggests that about half of the one plus year class does not mature short of the second fall migration. This, undoubtedly, explains part of the difference in growth schemes shown in Figure 4. It is instructive to note that the west coast species, *Loligo opalescens,* was reported to die shortly after breeding (McGowen, 1954; Fields, 1965; Hobson, 1965). Such mortality has not been demonstrated for *L. pealei,* but it is inconsistent with Verrill's growth scheme if it does occur.

As shown in Figure 4, the linear regression lines fit to the 5th, 50th and 95th percentile sizes intersect on July 16 close to the hatching size, 2 mm. mantle length (see Arnold, 1965). Without taking into account the changes in body proportions which occur soon after a squid hatches, one can use this extrapolation to suggest that the probable hatching date for the current year class squid used in this study was approximately July 9, 1967. If allowance is made for a relative increase in mantle length to total length soon after hatching, the probable hatching date would be a few days later. Surface water temperatures in Vineyard Sound ranged from 15 to 19° C. between mid-June and mid-July, 1967. A collection of freshly laid squid egg capsules taken in 14 m. of water northwest of Gay Head on September 19, 1967, hatched in 20 days in the laboratory at water temperatures of 16 to 18° C. Thus I estimate that the egg deposition which produced the current year class squid in this study took place about June 19, 1967.

TABLE I

Early growth rate of three loliginid squid

Species	Location	Month hatched	Growth rate, mm./month to given age	Reference
L. vulgaris	North Sea	VII	20 mm., 6 months*	Tinbergen and Verwey, 1945
L. vulgaris	Mediterranean Sea	VI or VII	16.3 mm., 3 or 4 months 13.6 mm., 5 or 6 months	Mangold-Wirz, 1963
L. opalescens	Monterey Bay, California	V	6 mm., 12 months	Fields, 1965
L. pealei	Vineyard Sound, Massachusetts	VI	30–48 mm., 1 month 25–34 mm., 2 months 20–27 mm., 3 months 16–17 mm., 4.75 months	Verrill, 1882
L. pealei	Menemsha Bight, Vineyard Sound, Massachusetts	VII	17.8 mm., 4 months†	Summers, this paper

* Tinbergen and Verwey reported ventral mantle lengths which are slightly shorter than the dorsal mantle lengths given by the other authors.

† This is the growth rate of the median, or 50th percentile size. The 5th and 95th percentile sizes have growth rates of 11.2 mm. and 27.5 mm. per month for the first four months, respectively. These three growth rates are illustrated by the linear regression lines in Figure 4.

Verrill gave the date of first significant hatching as "the second week in June." The difference between his date and the one given above may reflect a difference in years (1875 *vs.* 1967), different collecting sites, his acquaintance with Long Island Sound or a combination of these factors. Adult squid were abundant in Vineyard Sound by June 1, 1967, when several fishermen began catching them regularly. Sizable accumulations of squid egg capsules were taken in the Bureau of Commercial Fisheries fishtrap in Buzzards Bay on June 3 (Mr. C. L. Wheeler, personal communication) and from Falmouth Beach on June 17, 1967. Verrill's mid-June hatch seems distinctly early for the 1967 season.

I take these isolated collection of egg capsules and the narrowness of the current year class size distribution to be an indication that *L. pealei* breeding activity is highly localized and that the breeding season is made up of a number of these events. This is a logical consequence of Arnold's observation on the mating behavior of this species (1962), which indicated the importance of a visual stimulus in egg deposition.

The early growth rate of three loliginid squid is given in Table I. The first four entries are based upon modal analyses. With the exception of Fields' data, all of the entries are roughly comparable over the first few months of growth.

SUMMARY

1. This paper reports estimates of the growth rate and size distribution of current year class *L. pealei* based upon the population statistics of daytime, otter trawl collections in Menemsha Bight during the second half of 1967.

2. The selectivity of a #35 otter trawl with a 1.25-inch mesh (3.2-cm.) stretched measure cod end liner was estimated by a 50% retention of *L. pealei* with a dorsal mantle length of approximately 3.7 cm.

3. The current year class, summed older year classes and individual older year classes of *L. pealei* were found to have lognormal size-frequency distributions.

4. The current year class could readily be separated from the older year classes by the use of Cassie's method on lognormal probability paper.

5. The growth rate of a median individual in the current year class of 1967 was found to be 1.8 cm. dorsal mantle length per month for the first four months past hatching.

6. Approximately one-half of the one-year-old *L. pealei* did not mature sexually before the second fall migration. None of the current year class *L. pealei* matured before the first fall migration.

7. The results indicate that *L. pealei* egg deposition is isolated in time and location, and repeated throughout the Vineyard Sound area from at least June through September.

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FORM AND HABIT IN SPECIES OF MALLEUS (INCLUDING THE "HAMMER OYSTERS") WITH COMPARATIVE OBSERVATIONS ON ISOGNOMON ISOGNOMON

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Probably no tropical bivalve shells have been better known than those of the well-named "hammer oysters." The black species appears as *Ostrea malleus* in the 10th edition of the *Systema naturae* of Linnaeus. The genus *Malleus* was erected by Lamarck in 1799 for the inclusion of this species and later for the white *M. albus.* These shells were highly prized by early collectors; Dance (1966) records the sale of shells of *M. malleus* for 32 guilders in Holland and 240 francs in Paris during the eighteenth century. The distinctive, indeed exaggerated, hammer shape of these two species has not proved characteristic of the genus (as defined by Thiele (1935)) unless those smaller species without the anterior and posterior extensions of the hinge line be placed in separate genera, such as *Parimalleus* (Iredale, 1939).

Despite long knowledge of and interest in the shells, effectively nothing is known about the mode of life of any species of *Malleus.* Coming from coral reef areas they have been regarded as being attached to them. Thus Iredale (1939) in his account of the bivalves collected during the course of the Great Barrier Reef Expedition refers to *M. malleus* as an inhabitant of coral reefs but states that *M. albus* is not a reef shell but "the mainland species." Although some account of the structure of species of *Malleus* has been given by Pelseneer (1911) and Kühnelt (1938), no one appears ever to have observed these animals in life.

Personal opportunity of so doing was afforded during the sixth cruise of the Stanford University Research vessel "Te Vega" which the author accompanied from Singapore to the Solomon Islands from January to March, 1965. Some additional, and very valuable, information was obtained during some four weeks spent on the Belgian "De Moor" Expedition to the Great Barrier Reef of Australia during September to October, 1967. Acknowledgments for these facilities are given later.

Detailed examination of the smaller, *M. regula* (Forskål), without extended hinge line, provided the basis for conclusions on the adaptations and habitats of *M. malleus* (Linn.) and *M. albus* Lam., specimens of which were obtained from various sources and which undoubtedly represent successively further stages in adaptive evolution, essentially from epifaunal to infaunal life. The striking fact that *M. regula* may occur in vast numbers mingled with similar populations of *Isognomon isognomon* (Linn.), a species of a not very closely related genus which has also taken to vertical posture after byssal attachment, involved an illuminating parallel study. Both species exhibit most interesting consequences of the mono-

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[1] From the Te Vega Expedition, Cruise 6.

EXPLANATION OF LETTERING

A	anus
ABR	anterior byssal retractor
AD	adductor
AE	anterior extension of shell
AF	accessory foot
AOL	anterior outer ligament layer
AOM	epithelium secreting anterior outer ligament
AU	auricle
BG	byssal groove
BN	byssal notch
By	byssus
CT	ctenidium
E	exhalant current
F	foot
FL	fusion layer
I	inhalant current
ICL	inner calcareous layer
IF	inner fold of mantle margin
IL	inner ligament layer
LP	labial palp
MF	middle fold of mantle margin
MI	mantle isthmus
ML	multivincular ligament
N	limit of nacreous region
OBG	opening of byssal gland
OCL	outer calcareous layer
OF	outer fold of mantle margin
P	periostracum
PAR	pallial retractor
PBR	posterior byssal retractor
PC	pericardium
PE	posterior extension of shell
PMC	promyal chamber
POL	posterior outer ligament
POM	epithelium secreting posterior outer ligament
PR	prismatic layer
R	rectum
RI	ridge associated with pallial retractor
U	umbo
V	ventricle

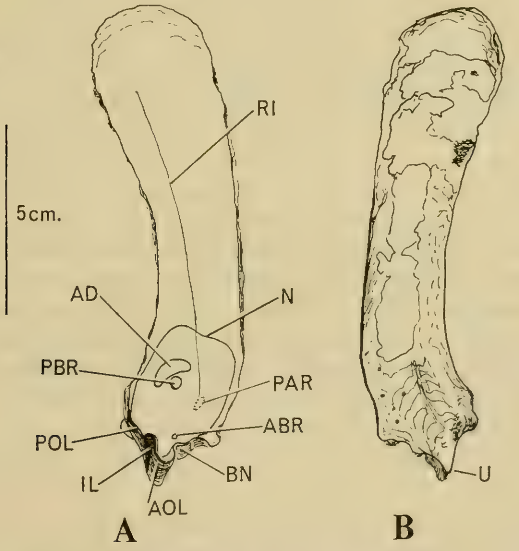

FIGURE 1. *Malleus regula,* right valve, **A**, inner, **B**, outer view. For explanation of lettering in this and all other figures see above.

myarian condition, description and discussion of which supplement a series of prior studies on other monomyarians.

The above statement explains the general plan of this paper in which descriptions of structure, illuminated where possible by discussion of function, precede an account of mode of life and general biology.

THE GENUS MALLEUS LAMARCK 1799

Malleus regula (Forskål)

This species, widely distributed and very common in the tropical Indo-Pacific, was first personally observed at Palau Gaya, one of a half-circle of three precipitous islands forming the remains of the rim of a large extinct, now flooded, volcanic crater in Darvel Bay on the east of Sabah (North Borneo) which was visited on the *Te Vega* at the end of January, 1965.

Shell

This is straight (Fig. 1), about four times as long as it is broad and attains lengths of some 12 cm. Old shells tend to be very irregular with the outer surface (Fig. 1B) usually covered with calcareous algae and other encrusting growths. It stands more or less erect, firmly attached by byssus threads to a rocky, often partially mud-covered, surface. The prominent posteriorly directed umbones (U) are situated immediately on the posterior side of the byssal notch (BN). These mid-dorsal structures thus lie in the middle of the topographically under surface. This is true of all the species considered (including *I. isognomon*), which for this reason are figured with the umbonal (*i.e.,* "dorsal"), as well as the byssal (*i.e.,* "ventral"), surface undermost as they invariably occur in life.

The internal surface of the shell valve is shown in Figure 1A, and that of *M. malleus* in Figure 3. The nacreous or proximal area (delimited by N) occupies some quarter of the surface, the distal remainder (apart from some thin superficial periostracum) consisting of prismatic (outer calcareous) layer formed by the mantle margins. Conditions are precisely as in *Pinna* (Yonge, 1953b). In both, the mantle is capable of great extension, and corresponding withdrawal, and is able to make rapid repair following the frequent damage species of both genera experience. Within the space occupied by the visceropedal mass (where the nacreous, *i.e.,* inner calcareous, shell layer is secreted by the mantle) lie the scars of the somewhat crescentic adductor (AD) and of the larger posterior and the smaller anterior byssal retractors (PBR, ABR). There is also the fainter scar of the pallial retractor (PAR). From this a low ridge (RI) runs along the prismatic (topographically upper) extension of the shell dividing this lengthways into anterior and posterior regions, corresponding, as shown in Figure 2A, to inhalant and exhalant regions, the former twice as wide as the latter.

Ligament

As shown in more detail in Figure 2A (also in figures of *M. malleus* and *M. albus*) this consists of a centrally placed convex inner layer (IL) secreted by the mantle isthmus (MI) with a shorter anterior, and a longer posterior, outer layer (AOL, POL) secreted in the embayments by the outer surface of the outer fold of the mantle margins. Progressive stages in the growth of the ligament are shown in Figure 2 indicating "ventralward" displacement of the hinge line which is characteristic of cemented bivalves [*e.g., Hinnites, Spondylus, Etheria, etc.* (Yonge, 1951, 1962)] and would seem to be due in the case of this byssally attached species to a similar close adherence of the hinge region to a rock surface. In other byssally attached bivalves, *e.g.,* the Mytilidae, the hinge is not pressed against the substrate.

The ligament is bounded anteriorly on the right valve by the deep byssal notch (Fig. 1, BN). Further reference to the ligament is made in the accounts of *M. malleus* and *M. albus*.

Mantle cavity

The appearance of the animal when fully expanded after removal of the left shell valve and the mantle lobe is shown in Figure **2A**. The extent of the nacreous layer within which lies the relatively small visceropedal mass is indicated by the broken line (N). The mantle lobes have extended above this carrying the ctenidia (CT) with them to fill the prismatic extension. Any stimulus causes contraction of the pallial retractor (PAR), the presence of which was first noted by Pelseneer (1911), and consequent withdrawal of the mantle lobes, if complete then within the confines of the nacreous region. There is no pallial line in these monomyarians. Of the three marginal folds, the outer one (Fig. 2B, OF), which secretes an extremely thin but very elastic periostracum (P) as well as the prismatic layer (PR), is obscured by the larger middle fold (MF) which bears a single row of small tentacles as does the better developed inner fold (IF) where they are alternately larger and smaller. By apposition or separation these inner folds, or pallial curtains, control entrance into or exit from the mantle cavity.

The lobes are nowhere fused although closely applied to one another at the tips of the ctenidia somewhat to the posterior side of the end of the distal extension (Fig. 2A). This represents the point of division between inhalant and exhalant chambers. The functional advantage of a wide inhalant region, well raised above the bottom and extending along both anterior and distal surfaces, is apparent.

Promyal chamber

In addition to the extensive exhalant chamber distal to the adductor, water also passes posteriorly by way of a promyal chamber (PMC) corresponding to that initially described in *Crassostrea* by Nelson (1938) and now realized as one of the distinctive characters of that genus compared with *Ostrea*. It represents a secondary connection, on the dorsal side of the adductor, between the anterior and posterior ends of the mantle cavity and, as in *Crassostrea,* runs along the right side. After passage through the ctenidia, the flow of water, along the course indicated by the broken arrows in Figure 2, is assisted by cilia lining the chamber and facilitated by displacement of the rectum to the left side as described and figured by Pelseneer (1911) although without knowledge of its significance. Extrusion is then direct (Fig. 2A, E_1) or, if the mantle margins are applied in this region, in more distal regions of the exhalant chamber.

Nelson pointed out that the promyal chamber in *Crassostrea* increased the pumping rate and also permitted life in greater concentrations of sediment. He correlated its presence with greater elongation in *Crassostrea* and consequent greater distance between the hinge line and the adductor compared with the more rounded *Ostrea*. All of these arguments apply equally to *Malleus,* particularly the last although, as noted later, there is no promyal chamber in the effectively equally elongated *Isognomon isognomon*. The chamber in *M. regula* and *M. malleus* is relatively smaller than in *Crassostrea* owing to the presence of the large

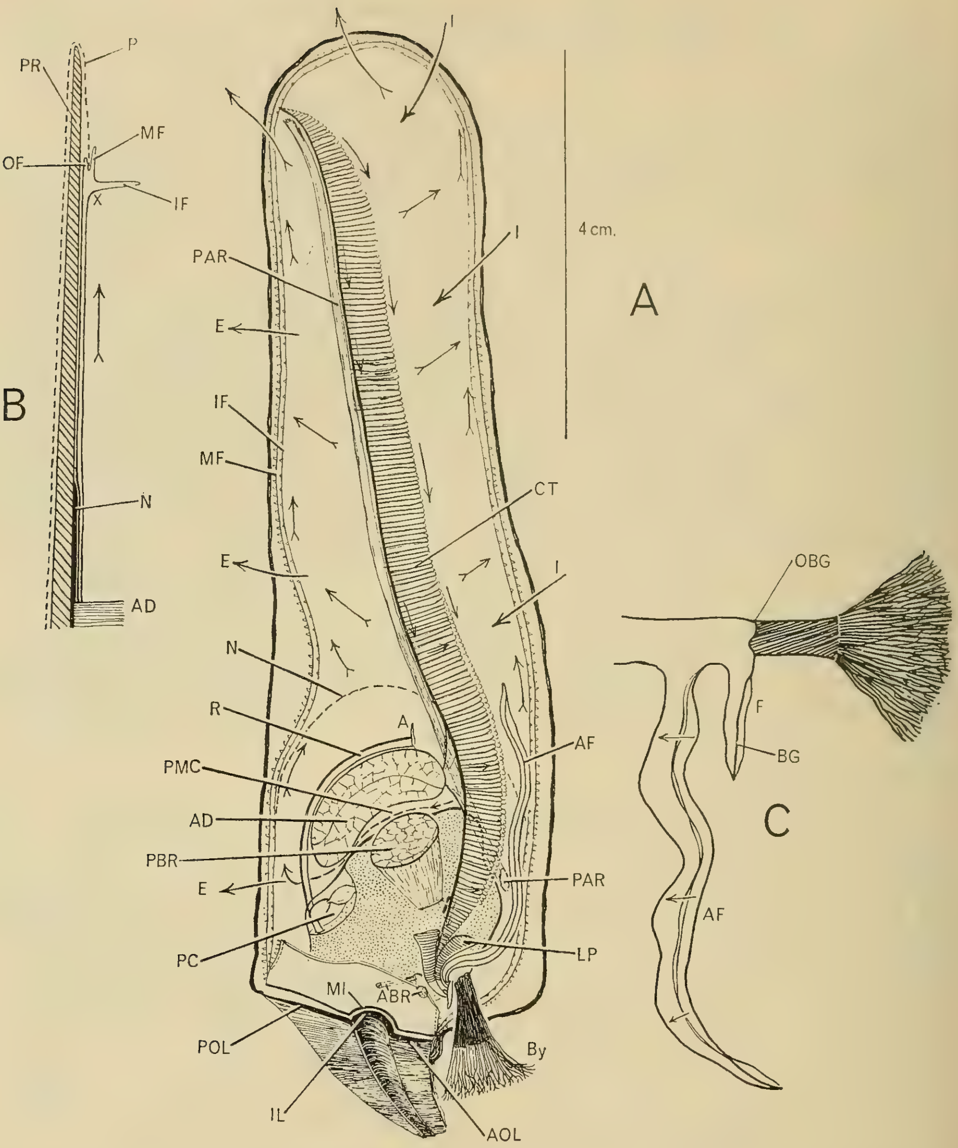
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FIGURE 2. *M. regula.* **A**, fully expanded animal within right valve, left mantle lobe removed. Plain arrows indicate direction of collecting currents on the ctenidia, also of inhalant currents; feathered arrows direction of cleansing currents. **B**. Section through valve and mantle distal to adductor showing three marginal mantle folds with periostracum (broken line), outer prismatic layer (oblique lines) and inner nacreous layer (black) of the valve. Waste material carried marginally and then, where marked with x, conveyed distally. **C**, foot, with byssal groove and byssus, and accessory foot viewed laterally. Arrows indicate direction of ciliary beat on extremely active accessory foot.

posterior byssal retractor (PBR) absent in the Ostreidae (and also in adult *M. albus*).

Ctenidia and palps

The greatly extended filibranch ctenidia are attached proximally by way of a suspensory membrane to the anterior surface of the adductor (Fig. 2**A**) and at the distal extremities to the inner fold of the mantle margins. Attachments between the inner demibranchs and between the outer demibranchs are all by way of interlocking cilia as in other Pteriacea including *Pinna* (Yonge, 1953b). The structure of the gill in *M. malleus* and *M. albus* is described by Ridewood (1903) and Atkins (1936, 1937, 1943) although, despite statements to the contrary, the ctenidium is heterorhabdic with principal filaments. There is fusion between filaments in the region of the "ventral" margins but not terminally. Cilia on the frontal surfaces beat "ventralward" except for those on the inner surface of the outer demibranch which beat towards the axis. There are five oralward currents, three "dorsal" and two "ventral," although there is only a groove along the margin of the inner demibranch. No evidence of antagonistic frontal currents, such as Atkins (1936) found in the related *Pteria,* was noted. There are both longitudinal muscles in the axis and lateral longitudinal muscles on either side as described and figured by Atkins (1943). They must function in co-ordination with the pallial retractor.

The rather small, rather elongate palps (LP) call for no comment. They are symmetrical on the two sides and without fusion of the lips such as occurs in certain Pectinacea. The mouth is relatively large.

Foot and retractors

As described and figured by Pelseneer (1911), the foot in *Malleus* is unique in being divided into two portions. Both project anteriorly and are ventrally grooved; the first is elongated, the more distal bears the byssal opening. The former, designated "bandförmigen Tentakel," was found by Kühnelt (1938) in *M. albus* and considered a possible taste organ. It is here termed the accessory foot (AF) and observations in life have extended knowledge and provided better understanding of possible function.

As shown in Figures 2**A** and **C**, the foot (F) is concerned exclusively with the planting of byssus threads which issue from the opening of the byssal gland (OBG) and are directed along the ventral groove. The result is the formation of an elaborate byssus (By), the threads consolidated basally to form a massive stalk. This is the sole function of this region in adult life. It could be concerned with locomotion in early post-larval life as it is in the Mytilidae; it retains that function throughout life in the closely allied *Pinctada* (Herdman, 1904). The bivalve foot may also be a cleansing organ as in certain cemented genera, *e.g., Spondylus* and the Chamidae (Yonge, 1967a) and most strikingly in the Anomiacea where the massive, calcified byssus is planted direct from byssal opening while the elongate foot is concerned exclusively with cleansing the mantle cavity (becoming locomotory again in the limpet-like *Enigmonia;* Yonge, 1957). In the Pinnidae the foot is solely concerned with byssal attachment but a unique pallial organ in the *exhalant* chamber probably clears that region, especially following the frequent damage these vertically disposed bivalves experience (Yonge, 1953b).

In *Malleus* the function of cleansing has been taken over by the extremely long and active accessory foot which moves with complete freedom throughout the lower regions of the inhalant cavity continually turning and twisting, extending and withdrawing. It is grooved "ventrally" and everywhere ciliated but owing to the constant movement it was impossible to follow the direction of beat except on the outer surface where it is always away from the groove. The function of the cilia appears to be to keep the foot clean, not to direct particles in any particular direction. Transverse sections reveal the presence of a thin layer of circular muscle and within this much greater amounts of longitudinal muscle divided into groups by inward-running strands of fine connective tissue. Blood sinuses are numerous, extension is clearly by blood pressure and contraction of circular muscle, retraction by contraction of the longitudinal muscle.

This is a unique structure which cannot be homologized with any part of the divided foot in the Gastropoda. Its presence is to be correlated with the vertical posture in *Malleus* and the danger of sediment accumulations in the restricted pocket at the base of the inhalant chamber with consequent blockage of palps and mouth (see Fig. 2A). Similar dangers to other similarly disposed anisomyarians, *e.g.*, the Pinnidae and *Pedum spondyloideum* which live, respectively, in sand and in cavities within reef-building corals, are met by way of the unique waste canal in the former (Yonge, 1953b) and of a powerful anterior cleansing current through the byssal notch in the latter (Yonge, 1967b). The small anterior and much larger posterior retractor muscles (ABR, PBR) are symmetrically disposed and effectively entirely concerned with the byssus. Movement of the accessory foot is by intrinsic agencies.

Cleansing

The general course of the cleansing currents in the mantle cavity is indicated by the feathered arrows in Figure 2A. All material in both chambers is carried distally by way of currents running along the inner side of the inner mantle fold (Fig. 2B) and ejected in the distal extremities; there is no accumulation of pseudofaeces in the inhalant chamber. Cleansing in the base of the inhalant chamber is effected by the accessory foot. Longish faecal pellets discharged from the anus (A) are also conveyed distally for disposal.

Visceropedal mass

Much of this has been described; other anatomical features (shown in Fig. 2A) include the posteriorly disposed pericardium (PC) with the rectum passing through the ventricle and both displaced to the left as described by Pelseneer (1911) in consequence of the development of a promyal chamber. The course of the alimentary canal is simple. The gonad covers most of the visceral mass although all specimens opened—in early February—appeared empty (unlike the associated *Isognomon*), implying recent spawning.

Observations on small individuals

Observations made on animals between 1 and 2 cm. long and up to 6 mm. wide were illuminating. Apart from the byssal notch on the right valve, they are equivalve, almost completely straight-sided and with the prismatic distal region completely plastic and readily bent in any plane. An instance was noted where the distal region had turned at an angle of 90° in the plane of the valves (it could equally be at right angles to this). The adaptive advantage to individuals which may settle under rocks and can only survive by growing around their margins is obvious. The end result is *always* that the distal regions point upward, opening to receive food and oxygen. This region is initially incapable of closure; contraction of the adductor pulls together the rigid nacreous, but not the distal prismatic, region. As in *Pinna,* this shell layer has a high proportion of conchiolin. However, accompanying withdrawal of the mantle lobes will effectively seal the nacreous region. On later relaxation the mantle lobes could be seen, through the translucent shell, slowly "creeping" back into the distal extension. It is probable that the periostracal sheet, which must be stretched during withdrawal, assists this process. Increased growth brings added danger of damage. However the distal shell is capable of indefinite repair (as in the similarly constructed Pinnidae and most strikingly in *M. malleus;* see Fig. 5**A**) so that larger shells become increasingly irregular while their full exposure leads to overgrowth by encrusting organisms.

Malleus malleus (Linn.)

This is the black hammer oyster, the longest known and best known species. During the cruise of the *Te Vega,* living specimens were obtained from shallow water near Zamboanga in the Philippines and through the kindness of divers from deeper water in the harbor at Rabaul, New Britain. Subsequently others were examined which had been collected by diving off Lizard Island and off Low Isles by Dr. A. Bastin of the *De Moor* Expedition. In all cases the animals came from relatively clean sandy bottoms in which they were largely buried, only the most distal regions projecting. Specimens measured in Rabaul were 18 to 21 cm. long with a "hinge line" up to 21 cm.; in Australia up to 22 cm. long by as much as 24 cm.

The interior of an adult shell is shown in Figure 3**E**. The general appearance is similar to that in *M. regula* with a relatively smaller nacreous region bearing the same four muscle scars. There is a well developed byssus but here *both* valves are notched, although to a greater depth on the right. The significant difference, apart from the greater extent of the prismatic region distally, is the formation of similar areas in line with the hinge to produce a very long posterior, and a somewhat shorter anterior, extension (PE, AE). It will be noted (Fig. 3) that the nacreous region is *not* concerned in this. The mantle lobes pass into and are withdrawn away from these extensions exactly as they do in the distal region. While the posterior extension does resemble (on an hypertrophied scale) the posterior auricle in a pectinid, this is not true of the anterior extension which is separated from the hinge and ligament by the byssal notch. There is thus no anterior extension of the ligament but this is not true posteriorly as revealed by the transverse sections—actually of *M. regula* but more suitably discussed in

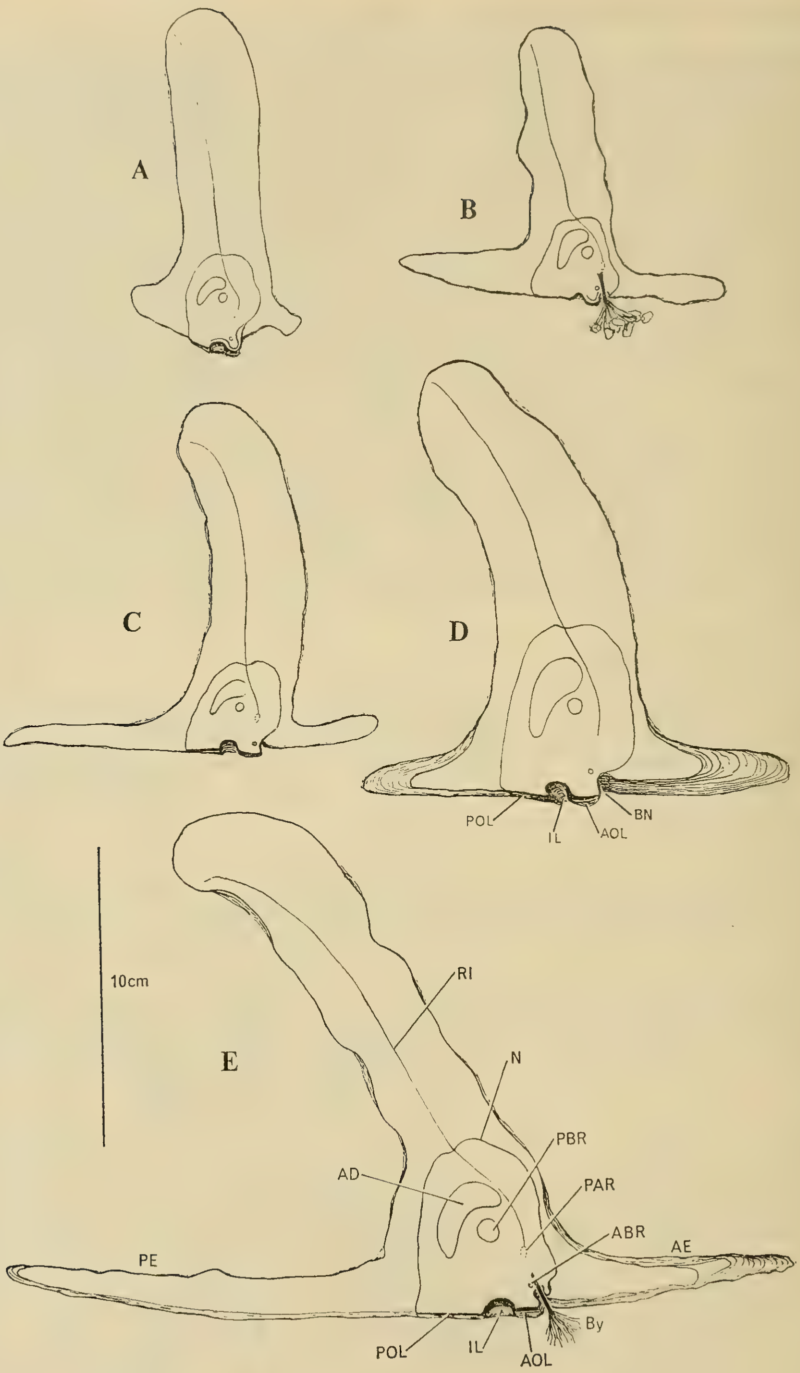
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Figure 3. *M. malleus,* **A–E**, interior views of right valves showing various stages in growth with full development of anterior and posterior extensions of the "hinge line" shown in adult **(E)**.

relation to *M. malleus*—shown in Figure 4. These indicate the structure successively from anterior to posterior (*a-g*) of the anterior outer, the inner and the posterior outer ligament layers (AOL, IL, POL) with their respective secreting epithelia (AOM, MI, POM). There is a posterior movement in growth indicated by the intrusion of the anterior outer layer into the inner layer (*b, c*) and a corresponding extension of the latter backward on either side of the posterior outer layer (*f*). Finally, posterior to the primary ligament formed by these three layers (with initially a covering periostracum) there is a secondary extension consisting of fusion layer (*h,* FL) formed by the union of the outer surfaces of the outer fold of the mantle margins, a secreting surface which elsewhere forms the outer calcareous (*i.e.,* prismatic) layer of the valves (OCL). This secondary ligament, short in *M. regula,* is of impressive length in *M. malleus* and *M. albus* (Fig. 6) owing to the great posterior extension of the hinge line in those species. It can have little or no functional significance. It corresponds to the long posterior secondary ligament in the Pinnidae (Yonge, 1953b) which is certainly functionally significant. Both represent the consequence of pallial fusion associated with posterior extension of hinge line.

Apart from the lateral extension of the mantle lobes, internal structure is similar to that of *M. regula.* Interest concentrates on the formation of the lateral extensions of the shell and on habits and life history. As shown in Figure 3, these extensions appear at varying periods in the life history. Thus specimen **A**, although longer than **B**, still has only short extensions. In **B** and **C** the shell has the hammer form and is being vigorously extended in all three directions. In **D**, however, while length increases the lateral pallial extensions have been withdrawn although without effect on the extent of shell earlier secreted. But the mantle could again extend and increase the length of the "hinge line."

The transition from the condition in *M. regula* to that in *M. malleus* clearly occurs during the life history. As discussed later, it permits a changed mode of life. Apart from its vertical disposition, *M. regula* is a typical byssally attached epifaunal bivalve. *M. malleus* is not; it lives in clean but usually coarse sand with the byssus attached to fragments on this (much as does *Pinna*). The anterior and posterior extensions to the hinge line assist in the maintenance of the shell which is always buried with only the distal quarter or less exposed. The extent of exposure is usually revealed by the presence of encrusting organisms absent from buried regions of the shell. The shell extensions provide an anchor within the sand in which this species lives. But the byssus persists throughout life.

The relatively thin shell, the three projecting extremities composed exclusively of prismatic layer with a high proportion of organic conchiolin, inevitably suffers extensive damage. It is rare to find a large shell which does not exhibit extensive areas of repair. An example (from Rabaul) is shown in Figure 5**A**. The animal has withdrawn from a broken extent of distal shell and formed a new one at a somewhat different angle to the basal extensions, the form of which has also been somewhat altered. Such changes may be in any plane and many adult shells are of grotesquely distorted appearance. The area occupied by the viscero-pedal mass is seldom, if indeed ever, affected. Conditions are precisely as in the Pinnidae, also byssally attached within soft substrates, similarly exposed to damage and with the same means of extensive and rapid repair of these exclusively pris-

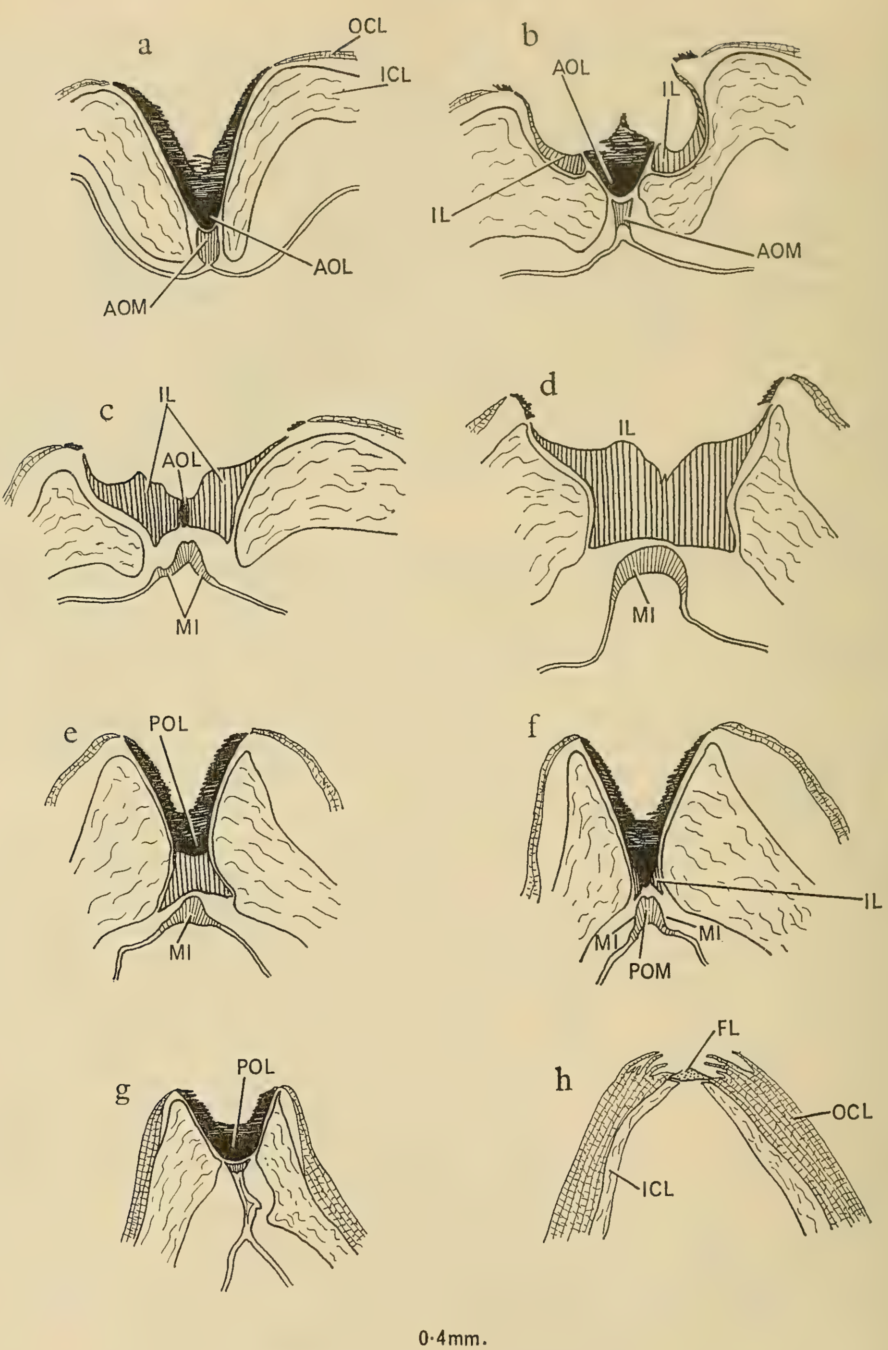
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FIGURE 4. *M. regula.* Transverse sections through ligamental region from anterior end back, *a-c* in region of anterior outer ligament; *b-f* in region of inner ligament; *e-g* in region of posterior outer ligament; *h* in region of fusion layer.

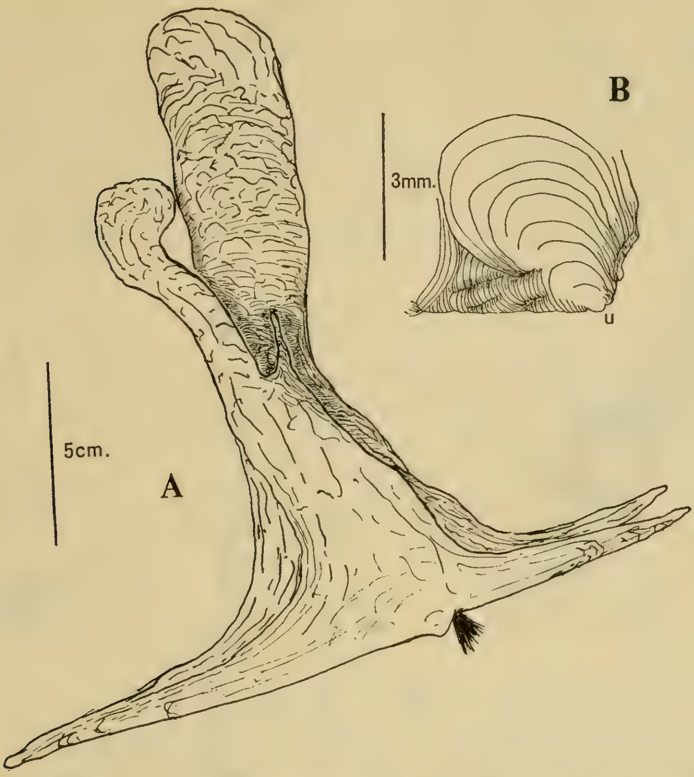

FIGURE 5. *M. malleus,* **A,** very irregular shell (from Rabaul); the mantle lobes have withdrawn from the first formed distal (prismatic) regions of the valves to form new ones at a somewhat different angle to the "hinge line." The nacreous region of the shell is not affected; **B,** umbonal region of left valve showing initial form (resembling *Pinctada*) immediately after settlement (After Jackson, 1890).

matic regions with very high conchiolin content (Bevelander and Benzer, 1948; Bevelander, 1952; Yonge, 1953b).

Malleus albus Lam.

The shell of this second hammer oyster (Fig. 6**A**) differs in being yellowish white and much stouter; the prismatic layer is more heavily calcified. There is less repair and the shells are therefore less irregular. They attain much the same size; the largest measured, from Bougainville in the Solomons, was 24.5 cm. long with a hinge line of 25 cm. Unlike *M. malleus,* the shell margins are deeply folded, the two valves interlocking very precisely. As originally noted by Lamarck, the adult shell lacks a byssal notch and Kühnelt (1938), in a description of a preserved specimen, found neither byssus nor posterior byssal retractor. As shown in Figure 6**A**, the corresponding muscle scar has disappeared, covered over by subsequent secretion of nacreous material.

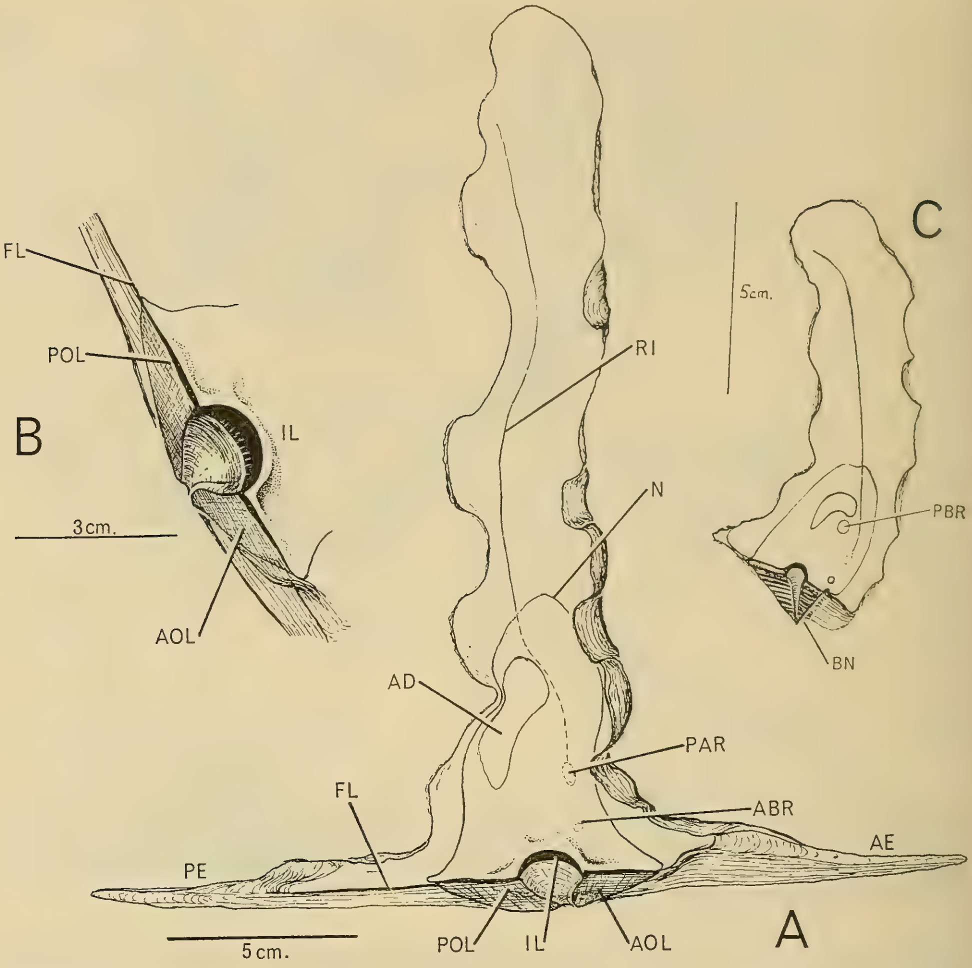

FIGURE 6. *M. albus.* **A**, interior of right valve of adult shell; **B**, ligamental region further enlarged; **C**, interior of right valve of half-grown specimen with byssal notch still present.

In a younger shell, at a stage before the hinge line has lengthened (Fig. 6C), there is both byssal notch (BN) and muscle scar (PBR). Unfortunately this shell, which was personally collected at Zamboanga in the Philippines, was empty. A living adult specimen was later collected by Professor T. F. Goreau by diving under the *De Moor* in the lee of Lizard Island within the Great Barrier Reef. The shell was 16.5 cm. long by some 12 cm. along the hinge line. There was no byssal notch, no byssus and no posterior byssal retractor although the small anterior muscle was retained. The true foot was reduced to a small protuberance with a minute opening of the former byssal gland and was situated at the base of the very large and extremely mobile accessory foot.

In *M. albus,* therefore, the animal comes to be anchored exclusively by way of the anterior and posterior extensions of the shell. Moreover it differs from *M. malleus* in living always in a substrate of muddy sand. Initial information to this effect was obtained from shell collectors at Rabaul; this was later confirmed by Mr. S. Bernik of Cairns, N.Q., who had collected specimens from muddy sand at depths of about 30 feet off Bougainville Island. The specimen from Lizard Island came from a similar substrate; it was lying somewhat obliquely with the distal third covered with sponge and other encrusting growths. Specimens of *M. malleus* from that area all came from clean sand.

Life History in the Genus Malleus

In the absence of precise knowledge any discussion about life histories must be on a basis of reasonable supposition. The problem is least in *M. regula;* there the larvae must settle on a rocky surface where mud is present. Unless impeded by overgrowths of rock the shell will then grow vertically. In the two other species metamorphosis of the settling veligers must be stimulated by contact respectively with a clean sandy or sandy gravel substrate or with one of muddy sand. Despite the opinion of Kühnelt, who considers the shell extensions as concerned with stability after attachment to rock, both *M. malleus* and *M. albus* are infaunal. Settlement of the larvae of *M. regula* on mud-covered rock could well represent the ancestral habit.

Initial byssal attachment is presumably to small fragments within the substrate. Gradual descent into this as growth proceeds must initially—as in the Pinnidae—be due to progressively deeper extensions of the foot with planting of new byssus threads on to fragments further down. The progressive extension of the hinge line will certainly anchor the animals more securely but at the same time must present an obstacle (absent in the anteriorly pointed Pinnidae) to the further penetration which certainly occurs. The eventual closure of the byssal notch in *M. albus* will even prevent any downward ejection of water which could assist further penetration in *M. malleus.* However a large shell of *M. albus* could well settle into the softer substrate it inhabits by its own weight. According to Mr. Bernik shells of this species are usually obliquely disposed with two regions of the shell protruding, presumably the posterior as well as the distal extremity. Professor R. D. Purchon (personal communication) states that, near Singapore, he collected living specimens lying on the surface of muddy sand. They could have been thrown up by storms. This does not appear to happen in the attached *M. malleus.* In both species not more than a third (often less) of the distal region projects. Differences in the two infaunal species are certainly associated with their distinct habitats. Water movements will be greater in regions where clean sand or shell sand accumulates. The combined effect of byssal attachment and shell anchorage would seem to be needed; the distorted form of larger shells indicates major disturbances and the means of coping with these. Water movements are obviously less where muddy sediments accumulate—so the byssus can be discarded while a thicker shell will oppose greater resistance to undue settlement of the massive adult shell into this softer substrate.

The Genus Isognomon Solander 1786

This genus, with *Malleus* included by Thiele (1935) in the Family Vulsellidae of the Pteriacea, differs from that genus in the possession of a multivincular type of ligament (Figs. 7, 8, ML). This has been described by Trueman (1954) for *Pedalion* (*Perna*) *alata.* (These generic names are synonyms for *Isognomon.*) Instead of the usual single inner ligament with bounding and superficial anterior and posterior outer ligament layers, the ligament is here subdivided into a series of inner ligament layers (or resilifers) with intervening outer ligament layers as shown in Figures 7–9. This type of ligament is shared with only the allied genus *Crenatula* and might be considered, as Trueman points out, to indicate wider than merely generic separation from *Malleus.* However the anatomical studies of Pelseneer (1911), which have been confirmed, do indicate close affinity between *Isognomon* and *Malleus* while Bernard (1898) describes the presence of a minute resilifer in association with the umbones and anterior to the large inner ligament in *Malleus.* By removing the major dissimilarity, this could indicate considerable affinity between *Malleus* and *Isognomon.*

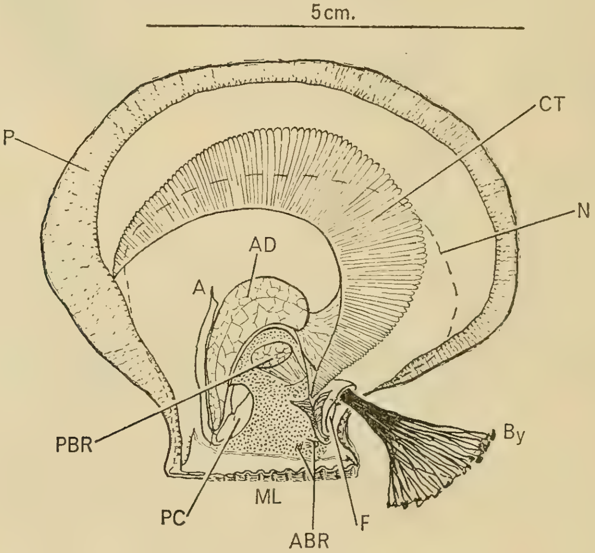

Figure 7. *Isognomon ephippium,* general view of animal lying within right valve, left valve and mantle lobe removed. Withdrawal of periostracal sheet formed by outer fold of mantle margin indicated (P).

Isognomon ephippium (Linn.)

A brief account is given of this species (Fig. 7), specimens of which were collected at Kieta on Bougainville Island. The rounded "oyster-like" form is characteristic of the majority of species of this genus which is widely distributed in the tropical Indo-Pacific and Atlantic (where it extends to Bermuda). This description forms a necessary preliminary to the more detailed account of *I. isognomon* where form and habit so closely parallel those in *M. regula.*

This and other rounded species are characteristically attached to rocky, often inter-tidal, surfaces by a massive byssus. This projects through a notch on the right valve on which side the animal lies precisely as do species of the pearl oysters, *Pinctada* and *Pteria* (Pteriidae). The animal has the typical monomyarian form with the viscero-pedal mass reorganized in relation to the centrally placed, although somewhat dorso-ventrally extended, adductor (AD) around which curl the ctenidia (CT). Although the nacreous region (delimited by the broken line N) is relatively larger than in *Malleus,* it is surrounded by a broad marginal prismatic region. The mantle margins retract across this retaining attachment to the edge of the shell by a delicate periostracal sheet (P). The small foot (F) is concerned, probably exclusively, with planting of the massive byssus (By). There are large posterior and small anterior pedal retractors (PBR, ABR) the latter with divided insertions. As shown by Pelseneer (1911) there is no asymmetry in the heart region, *i.e.,* no promyal chamber. As in all such monomyarians there is an extensive inhalant, and a correspondingly restricted exhalant, region. Ciliary currents were not particularly observed in this species; they doubtless correspond to those figured for *I. costellatus* by Stasek (1963) showing rejection of waste particles from both inhalant and exhalant chambers at a common point just on the exhalant side of the tip of the ctenidia.

Isognomon isognomon (Linn.)

This common Indo-Pacific species differs from *I. ephippium* and similar species in being elongated, attached in an erect position and, as noted already by Kühnelt (1938), assuming both the external appearance and mode of life of *M. regula* with which it is found. It is somewhat the larger, attaining lengths of 15 cm. and being somewhat broader. Appearance in life after removal of the left valve and mantle lobe is shown in Figure 8 which is directly comparable with that of *M. regula* (Fig. 2**A**.) There is obvious difference in the structure of the ligament which in *Isognomon* extends along the entire dorsal surface of the shell and in the position of the byssal notch, here anterior instead of dorsal. Consequently the massive byssus, the threads of which are not basally fused, emerges in that direction although applied to an under surface. The nacreous area is relatively much larger and (*cf.* Figs. 3 and 9) its margins less definite, the mantle lobes with the ctenidia not retracting so far. There is no pallial retractor and no corresponding ridge within the distal region of the shell and no promyal chamber. Without the accessory appendage, the foot is primarily concerned with byssal attachment but still large enough to have possible cleansing functions within the anterior extremity of the mantle cavity.

Distal to the nacreous region the mantle lobes and ctenidia are increasingly pigmented and finally dense black. The mantle margins are as in *Malleus* but thicker and relatively larger with short tentacles on the inner and middle folds. The non-plicate ctenidia are less firmly united and the filaments separate more readily (*i.e.,* are more typically filibranch) than in *M. regula.* As in that species there is no "ventral" groove along the outer demibranch and ciliation is similar. The tips of the ctenidia are attached to the inner mantle folds in the same position as in *Malleus,* the mantle cavity being similarly divided; the exhalant chamber is correspondingly greater than in the rounded *I. ephippium* (*cf.* Figs. 7 and 8).

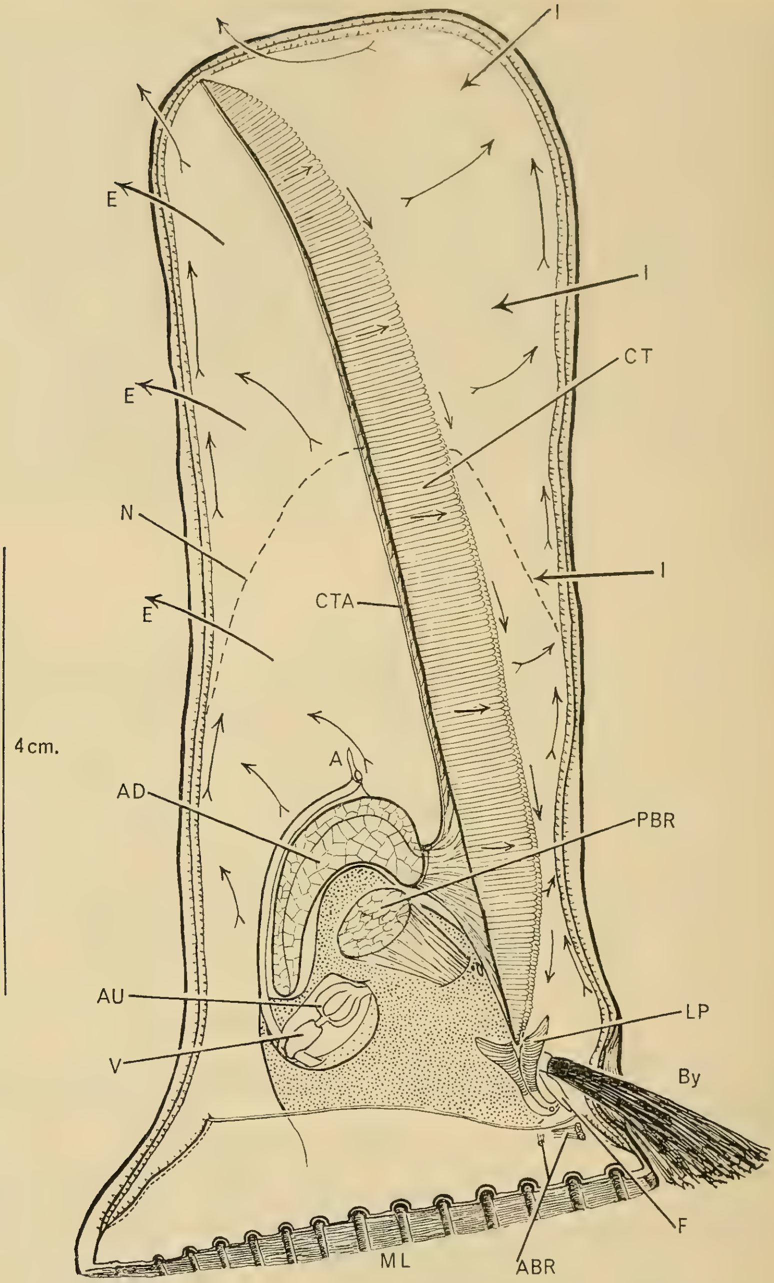
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FIGURE 8. *I. isognomon,* fully expanded animal within right valve, left mantle lobe removed. Plain arrows indicate direction of collecting currents on the ctenidia, also of inhalant and exhalant currents; feathered arrows direction of cleansing currents.

The palps are relatively longer than in *M. regula;* the lips are similarly developed, without frilling or fusion. There is a long anal flap which assists in directing the faeces, long scroll-like pellets up to 5 mm. long. As in *Malleus,* all waste material in both inhalant and exhalant chambers is carried to the inner edge of the inner mantle fold and so to the posterior distal regions where it is ejected (see feathered arrows in Figure 8).

Unlike *M. regula,* the gonad, which covers the visceral mass, was obviously maturing although sperm was not ripe.

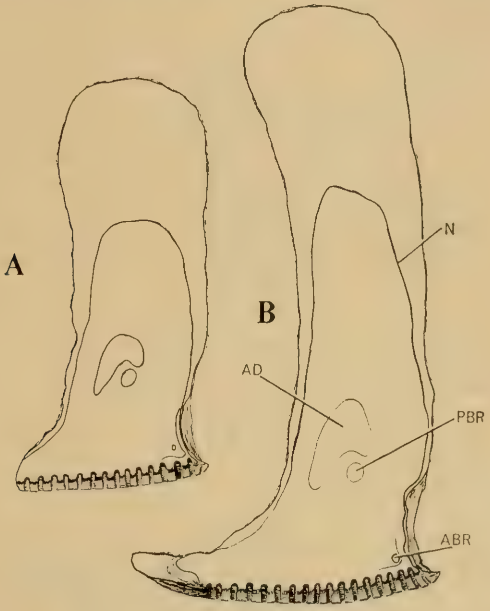


FIGURE 9. *I. isognomon,* **A, B,** interior views of right valves of two specimens showing stages in extension of the ligament and hinge line and of the distal region. The shells are 12 and 17.5 cm. long.

In the smallest animals collected the shell was 13 mm. long by 8 mm. wide and was oval rather than elongated like similar-sized *M. regula.* The shell is usually bluish with light-colored radiating curved rays. Young shells in dark areas tended to remain white. In some the foot was observed extended and seeking a surface for byssal attachment; when found initial threads were quickly secured. Until some 30 mm. long the shells remained oval and then displayed increasing irregularities due to the confined space in which they grew and to the effects of repair. But there is increasing elongation as form changes from the initially more rounded condition. They come to assume a striking external resemblance to *M. regula* although the hinge line is extended posteriorly to a greater extent than in that species (*cf.* Figs. 1 and 9). This process of extension continues until, as shown in Figure 9B, it may attain a length double the width of the shell. At this size the mantle tissues tend to withdraw, the ligament no longer elongates and the shell only increases in thickness.

Multivincular ligament

Two stages in the growth of the ligament are shown in Figure 11**A**, **B**. It begins as a simple opisthodetic ligament (**A**), the inner ligament layer inclined posteriorly from the umbones as in *Malleus*. But instead of this increasing steadily in size by added secretion from the mantle isthmus as in that genus (see Fig. 2**A**), new areas of inner ligament, or resilifers, appear at intervals posteriorly. Two of these are shown in Figure 11**B**; the maximum number observed in *I. isognomon* was 18. While obviously the number is related to the length of the hinge line, it is not necessarily related to the length of the shell.

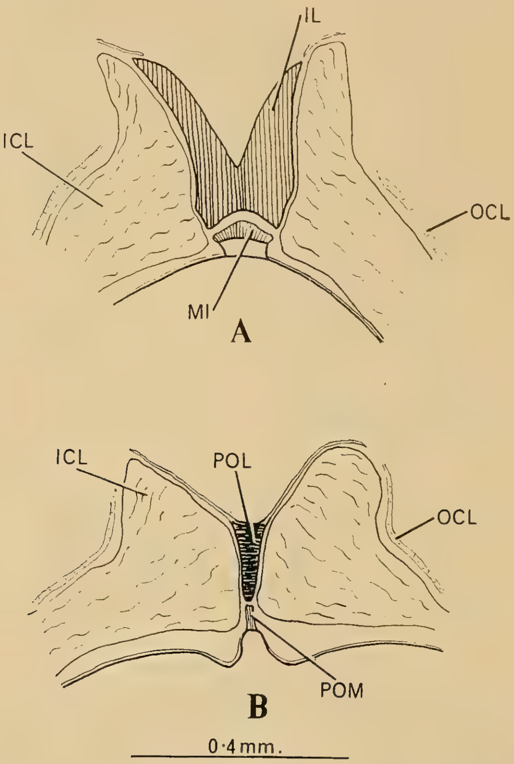


FIGURE 10. *I. isognomon,* transverse sections through ligament, **A**, through a region of inner ligament (a resilifer); **B**, region of outer ligament between resilifers.

In older individuals the original resilifer may be partially worn away or lost. Trueman (1954) describes a fusion layer but no sign of this appears in sections nor, in view of the absence of any extension of the mantle behind the primary ligament, as occurs in *Malleus* and *Pinna,* would its presence seem possible. The unsolved problem in this type of ligament is the origin of the successive areas of inner ligament, the one first formed being secreted by the mantle. Although there is as yet no certain supporting evidence, it appears most probable, as indicated diagrammatically in Figure 11**C**, that successive regions of the inner mantle surface (which normally secretes the inner calcareous, *i.e.*, nacreous, layer of the valves) fuse in the mid-line to invade the epithelia secreting the outer ligament layer and produce successive mantle isthmuses which secrete resilifer 2, then 3 and so on posteriorly. The inner mantle epithelium is here assumed to have the potential of secreting inner ligament should it, like the original mantle isthmus, become situated in the mid-dorsal line, *i.e.*, when subjected to the opening and closing movements of the valves. As in *Malleus* there is a marked ventralward movement of the hinge line as shown in the transverse sections (Fig. 10).

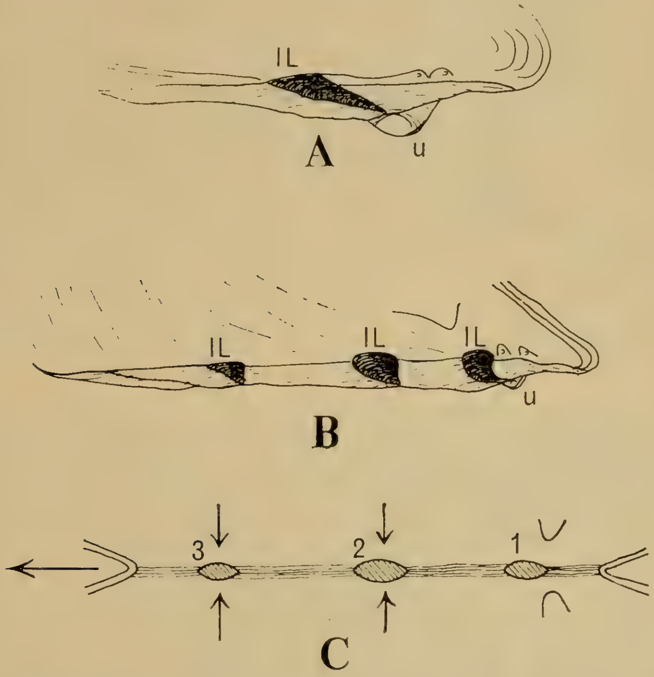
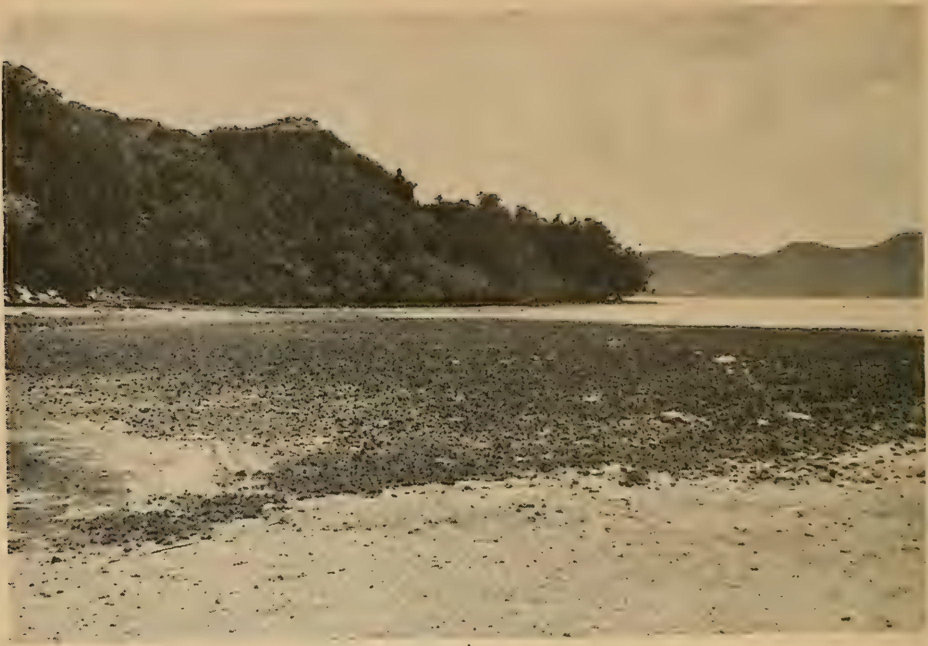


FIGURE 11. *I. ephippium,* views of ligamental regions of young shells (right valves), **A**, with only the original region of inner ligament; **B**, with two others formed subsequently (both from Jackson, 1890); **C**, diagram indicating possible mode of formation of multivincular ligament, 1–3 successively formed resilifers (1 in association with umbones), arrows indicate regions of possible cross fusion of inner mantle epithelium, horizontal arrow direction of ligamental growth (original).

Mixed Natural Bed of M. Regula and I. Isognomon

A remarkable mixed population of *M. regula* and *I. isognomon* was encountered in the narrow and very shallow channel between Palau Gaya and the much smaller Palau Tatagan. The general appearance of this "clam bed" at low spring tide is shown in Figure 12**A** with a closer view in Figure 12**B**.

The shallow rocky channel was everywhere covered with the densest possible growth of mixed *M. regula* and *I. isognomon.* The upright shells formed a compact "pavement" over an area of some 65 by 40 meters kept free by water move-



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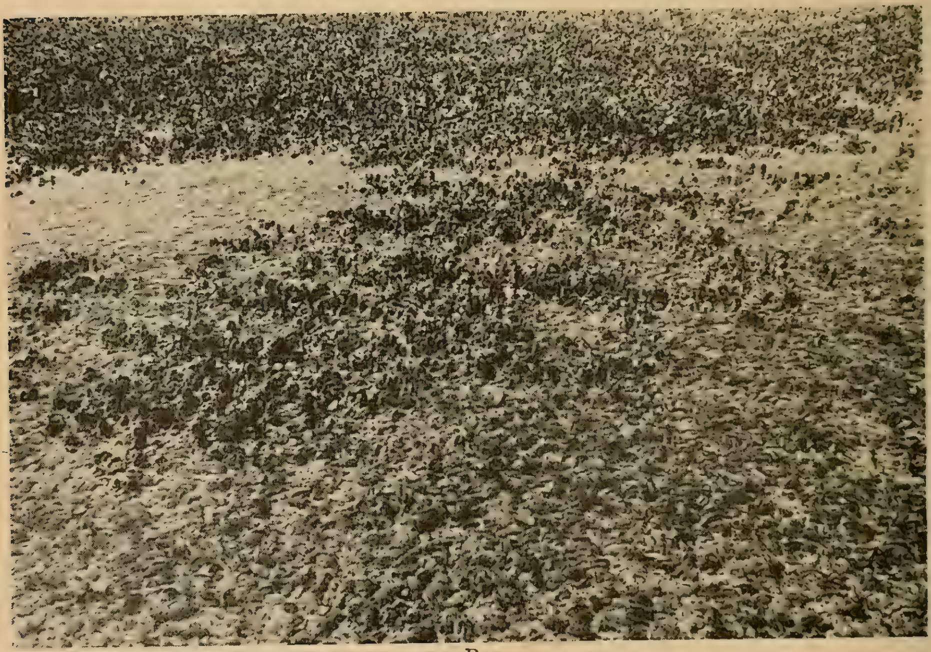

B

FIGURE 12. **A**, passage between Palau Tatagan and Palau Gaya photographed from south at low spring tide and showing exposed bed of mixed *Malleus regula* and *I. isognomon;* **B**, closer view of an area of the bed largely exposed and showing density of the mixed population.

ments from mud or sand. The maximum population counted within a foot square patch was 97, the largest *M. regula* being 12 cm. and *I. isognomon* 15 cm. long. The shells were usually irregular, due to dense crowding and frequent repair, but all pointed upward. Although the rock surface was irregular, all was smoothed out by the zonal growth of these bivalves above which occasional rocky patches projected.

As the tide exposed them sudden contractions of the "quick" muscle of the adductors produced innumerable jets of water (Fig. 12B). These ejections could be cleansing or be related to stages in mantle withdrawal. A final tightening up due to contraction of the "catch" muscle and the shells remained firmly closed until again covered with water.

Examination of samples indicated a greater proportion of *Malleus* in higher, and of *Isognomon* in lower, levels. In areas first uncovered proportions were almost 4/1, in mid and lower levels they were almost 1/1 but in the deepest level sampled, they were 1/3. Three factors could account for these slight differences in horizontal distribution; (1) tolerance to exposure (*i.e.,* effects of temperature and desiccation); (2) tolerance to presence of mud; (3) level at which the larvae settle. However the striking fact is not these minor differences but the complete mingling of two distinct species in the one habitat.

The bed constituted an interesting ecosystem. Within the shelter provided by the upright shells was a varied community of attached and free-living animals such as mussels, small cowries, crabs and weed with numerous ophiuroids moving actively between the bases of the shells. Occasional *Pinna* sp. were found in this unusual environment. Attached to the occasional patches of exposed rock was a distinct population of oysters, patelloid limpets, *Chama* sp., etc.

Evolution Within the Pteriacea

This group is more open to criticism than are the other four superfamilies—Mytilacea, Pectinacea, Anomiacea and Ostreacea—into which Thiele (1935) divided the Order Anisomyaria. Of the three constituent families, two genera of the Vulsellidae possess a multivincular ligament (but see Bernard, 1898), and so might be included in a separate family, while the heteromyarian Pinnidae have many unique features (Yonge, 1953b) and could be separated as a distinct Superfamily. However, accepting the present position, in Table I are listed certain crucial characters of the constituent genera with particular reference to habitat.

The Pteriacea occupy three distinct habitats: (1) a certainly primitive one byssally attached to rock or other hard surfaces, and secondary ones (2) without byssus within sponges (and also algae) and (3) with byssus in soft substrates although with eventual loss of this in *M. albus.* There is thus a tendency, absent in all other Anisomyaria, for return to infaunal life (the exception would be rock-boring Mytilacea, *e.g., Botula* and *Lithophaga*). It could be noted in possible defense of the present classification, that both the alivincular *Vulsella* and the multivincular *Crenatula* live in sponges, and both *M. malleus* with *M. albus* and the Pinnidae occupy soft substrates.

These points are illustrated in Figure 13. From the primitive habit of byssal attachment with the animal lying on the right side found in the Pteriidae (*e.g., Pinctada*) and also in most species of *Isognomon* (**A**) there is change to the vertical posture found in *I. isognomon* (**B**) and *M. regula* (**C**). From the latter condition comes invasion of soft substrates with accompanying elongation of the hinge line in *M. malleus* (**D**) and *M. albus* (**E**), the second losing the byssus in adult life. From both (**A**) and (**B**) there is independent change to life within sponges (**F**, **G**) with loss of the byssus. Finally, and independently, there is the infaunal habit of the Pinnidae (**H**), heteromyarians with an exceptionally large byssus.

TABLE I

Pteriacea

Family	Genus	Ligament		Byssus	Habitat		
		Alivincular	Multivincular		Epifaunal to rock, etc.	Infaunal sponges	Infaunal soft substrate
Pteriidae (Monomyarian)							
	Pteria	x	—	x	x	—	—
	Pinctada	x	—	x	x	—	—
Vulsellidae (Monomyarian)							
	Isognomon	—	x	x	x	—	—
	Crenatula	—	x	—	—	x	—
	Foramelina	x	—	x	x	—	—
	Vulsella	x	—	—	—	x	—
	Malleus regula	x	—	x	x	—	—
	M. malleus	x	—	x	—	—	x (coarse sand)
	M. albus (adult)	x	—	—	—	—	x (muddy sand)
(Pinnidae) (Heteromyarian)							
	Pinna	x	—	x	—	—	x
	Atrina	x	—	x	—	—	x

Elongation in Malleus and I. isognomon

The typical monomyarian (Yonge, 1953a) is rounded with the exception of occasional species or varieties in the cemented Ostreacea and Etheriidae (Unionacea), *e.g.*, respectively, *Crassostrea gigas* and *Etheria elliptica* var. *cailliaudi* (Yonge, 1962), although in both, and especially in the latter, it is the ligamental region particularly which elongates dorso-ventrally. Among those byssally attached, elongation is confined to *Malleus* spp. and to *I. isognomon*.

The primitive condition in the Pteriacea (omitting the Pinnidae) is probably best represented by *Pinctada* and by rounded species of *Isognomon*. Unlike the Pectinacea, the visceral mass is proximal to the adductor into a dorsal embayment in which its fits (Figs. 2A, 8). Distal extension is thus readily attained by change in growth gradients involving straightening and extension of the ctenidia. The difference between *I. ephippium* and *I. isognomon* (*cf.* Figs. 7 and 8) is brought about in this way and is associated with change to a vertical posture and a modified mode of life.

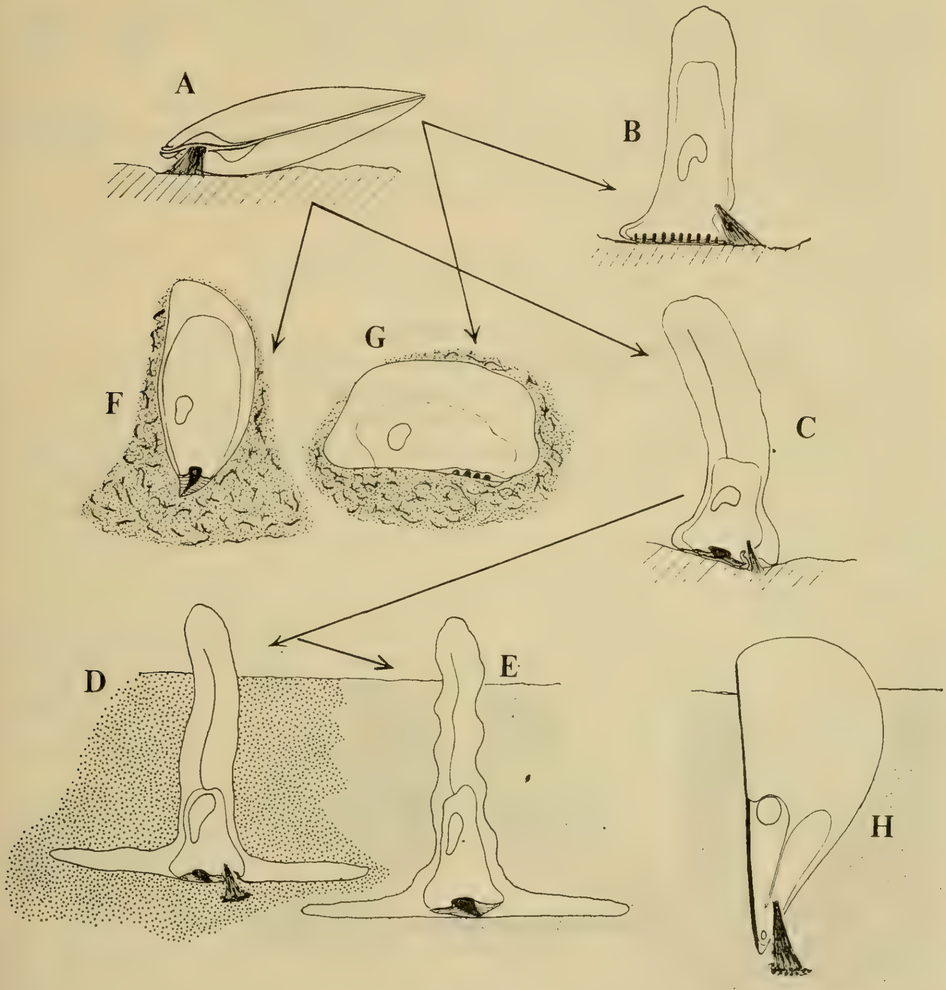

FIGURE 13. Pteriacea: drawings illustrating major adaptations and habitats of representative genera and species. **A**, *Pinctada* or *Isognomon ephippium* (alivincular and multivincular ligaments, respectively), rounded lying on right valve, byssally attached to hard substrate; **B**, *I. isognomon,* elongated, vertically disposed, byssally attached; **C**, *M. regula,* as in **B**; **D**, *M. malleus,* elongated with long hinge line, byssally attached within coarse sand; **E**, *M. albus,* as in **D** but adult not attached and in muddy sand; **F**, **G**, *Vulsella* (alivincular ligament) and *Crenatula* (multivincular ligament) both within sponges; **H**, *Pinna,* elongated, byssally attached in soft substrates.

Elongation is characteristic of the genus *Malleus.* Although, as shown in Figure 5B, the first formed shell is rounded, this is quickly altered by subsequent extensions of the mantle. The major changes involved consist of:

1. Great distal extension of the marginal prismatic zone of the shell.
2. Appearance of a pallial retractor closely associated with the elongated ctenidium.
3. Movement of byssal notch to the hinge line so that byssus emerges "downward."
4. Appearance of a promyal chamber proximal to the adductor (as in *Crassostrea*) made possible by elongation and permitting more efficient water circulation in the proximal region of the mantle cavity.
5. Development of a unique accessory foot concerned with cleansing of this region of the elongated mantle cavity.
6. Loss of any previous asymmetry associated with the recumbent posture, *i.e.,* of valves, byssal notch (gradually) and pedal musculature.

Other changes, notably elongation of the hinge line and eventual loss of the byssus, are associated with change from epifaunal to infaunal life.

Comparison between *M. regula* and *I. isognomon* has already been made (*cf.* Figs. 2 and 8). There is remarkable convergence both in form and consequent habit leading to the complete mingling of populations observed in the channel between Palau Gaya and Palau Tatagan. They are sympatric species. While the certainly more highly specialized *M. regula* might be expected to be the more successful this is certainly not the case at lower levels of the bed, over all levels of which *I. isognomon* occurs. The vertical distances are, of course, very small.

This mingling of populations of two species apparently contradicts the "competitive exclusion principle" of Gause (1934) which postulates that closely related (in this case similarly adapted) species with the same ecology cannot live together. Mayr (1963) has pointed out the exceptions, namely where two species occupy different niches in the same general habitat and the case of generalized herbivores where numbers are controlled by predators or disease and *not* by limitation of food. This could certainly apply to *M. regula* and *I. isognomon.*

The two species feed in identical ways and in shallow water where phytoplankton should be rich. Although *M. regula* is certainly the more highly adapted for dealing with collection of sediment within the vertically disposed mantle cavity this does not appear to give the species any obvious advantage over *Isognomon.* (It may well be a factor in permitting related species to exploit infaunal life.) As already noted, possible differences in settlement behavior and in resistance to limited degrees of exposure (but to *very* high temperatures) could account for the greater abundance of *M. regula* at higher, and of *I. isognomon* at lower, levels. But the major factor permitting co-existence of the two species would seem to be their different spawning seasons for which adequate evidence was obtained. In February *M. regula* had recently spawned but *I. isognomon* was only preparing to do so. There could be a gap of a month, or months, between the two events. There would therefore be no competition during the most crucial stages in the life history, when the larvae are planktonic and, above all, when they are settling with the accompanying, usually very rapid, metamorphosis into the adult form with assumption of the adult habit.

It is a pleasure to express gratitude to Stanford University for the invitation to participate in Cruise 6 of the *Te Vega,* itself made possible by N.S.F. Grant G17465. Particular thanks are due to Dr. Lawrence Blinks, then Director of the Hopkins Marine Station, Pacific Grove, who was in scientific charge of that cruise, and to Dr. Dan Cohen and to all others on board. Observations were extended during September to October, 1967, during the Belgian *De Moor* Expedition to the Great Barrier Reef of Australia thanks to the invitation of Professor M. Dubuisson, Rector of the University of Liège, and the kind co-operation of the Leader of the Expedition, Professor A. Distèche and his colleagues and also of Professor T. F. Goreau, a co-worker and fellow visitor. All microscopic sections have been prepared and all figures produced by the author's research assistant, Miss J. I. Campbell, whose help has been indispensable. Dr. Robert Robertson kindly read the proofs.

SUMMARY

1. Study of the structure and mode of life of *Malleus regula* provides the basis for consideration of the "hammer" species, *M. malleus* and *M. albus.*

2. *M. regula* occurs byssally attached, vertically disposed on rocky substrates associated with mud and is widely distributed in the tropical Indo-Pacific.

3. The distal two-thirds of the elongated shell is exclusively prismatic. By means of special pallial retractors the mantle lobes can be withdrawn within the nacreous region.

4. The massive opisthodetic ligament has a short secondary extension of fusion layer.

5. A promyal chamber on the right side proximal to the adductor increases water flow into the exhalant chamber.

6. The long filibranch ctenidia provide a vertically extended food-collecting surface.

7. The foot is concerned with planting of the massive byssus which emerges through a notch in the right valve but on the under (*i.e.,* dorsal) surface. There is also a unique and very long accessory foot, ventrally grooved, everywhere ciliated and in constant writhing activity due to blood pressure and intrinsic muscle. Moving freely throughout the lower mantle cavity it can only be concerned with cleansing.

8. Pseudofaeces are ejected from the distal tip of the mantle cavity.

9. *M. malleus,* the black hammer shell, occurs vertically embedded in coarse sand or sandy gravel. Byssus threads are attached to fragments within the substrate. The great anterior and posterior elongations of the hinge line (also exclusively prismatic) are separated by the byssal notch (now affecting both valves).

10. Shells are usually excessively irregular due to the great exposure to damage and the almost unlimited powers of rapid repair by the three pallial extensions.

11. *M. albus,* the white hammer shell, is stouter and inhabits muddy sand. During growth it loses the byssus with reduction of the foot and byssal retractors and closure of the byssal notch. The accessory foot is not affected. The animal becomes anchored in the substrate exclusively by the anterior and posterior extensions of the shell.

12. Description of the typically rounded *Isognomon ephippium* leads to that of the elongated *I. isognomon* which occupies precisely the same habitat as *M. regula*.

13. It lacks the pallial retractors, promyal chamber and accessory foot of *Malleus,* the nacreous region is more extended distally and the ligament is multivincular (the formation of which is discussed), producing some posterior, but never any anterior, extension of the hinge line.

14. A remarkably dense mixed bed of *M. regula* and *I. isognomon* in Darvel Bay, Sabah (Borneo), is described. Different spawning periods, by preventing competition during settlement, may account for this complete intermixing of sympatric species, which, however, are generalized herbivores where numbers are not limited by food supply.

15. From a basic epifaunal habit (*Pteria, Isognomon, M. regula*), members of the Pteriacea have become adapted for infaunal life within sponges (*Vulsella, Crenatula*) or within soft substrates (*M. malleus, M. alba* and the Pinnidae).

16. There is final discussion about elongation in monomyarians, *i.e.,* in the genus *Malleus* and in *I. isognomon.*

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1968

ABSTRACTS OF SEMINAR PAPERS

JULY 16, 1968

Sulfonation of rat lens proteins. SEYMOUR ZIGMAN.

When rat lens proteins were sulfonated using Bailey's reagent (Na_2SO_3, $Na_2S_4O_6$, 8 M urea, pH 9.9, HCO_3^- buffer), and then were subjected to preparative high voltage electrophoresis on Pevikon (polyvinyl chloride) plates (1.2 mm. thick) at pH 9.9, two components were obtained for both gamma crystallin and albuminoid. The mobilities of the faster-moving components (I) of both were 16 cm./hr. at 3000 v. as compared to 11 cm./hr. for the slower moving components (II). By eluting the proteins from the Pevikon with water, component I was present in both sulfo-protein fractions at twice the level as that of component II when 20-mg. samples were used. The recovery was approximately 80%.

Ultracentrifugation indicated that all four components had a 2.55 sedimentation rate. From amino acid analysis a molecular weight of 21,500 was found for each. Alanine was found to be the N-terminal amino acid of all four polypeptides. Immunochemical comparisons by precipitin test indicated cross reactivity among all four components.

Ultraviolet light extinction coefficients ($E_{1\%}^{1\,\mathrm{cm}}$) were 17–18 for components I, and 20–21 for components II. These differences were in agreement with the amino acid compositions of the components which showed tyrosine levels of components II to be 20% greater than those of components I. Lysine levels of both I's were nearly 2 × those of the II's; alanine levels of the I's were 1.5 × those of the II's.

By vertical acrylamide gel electrophoresis, sulfo-γI, sulfo-γII, and sulfo-albuminoid II gave very similar patterns of 2 close bands each. However, sulfo-albuminoid I exhibited many additional bands above these two (similar to α-crystallin).

The data, including a mathematical analysis of amino acid compositions, indicate that the albuminoid of the young rat lens is composed of approximately 66% γ-crystallin and 33% α- (perhaps in the subunit form). Interactions between γ- and α-crystallins leading to an insoluble product could involve all types of protein-protein binding, and may explain species and aging differences in the makeup of albuminoid.

Supported by the Rochester Eye Bank and PHS grant #NB 03081.

Possible role of protein synthetic enzymes in the control of protein synthesis in higher organisms. AUDREY E. V. HASCHEMEYER.

Comparative studies on aminoacyl-tRNA synthetases and aminoacyl transferases have been made in rat, frog (*Rana catesbeiana*) and toadfish (*Opsanus tau*). Enzyme activity levels in toadfish liver have been measured in relation to the temperature of acclimation of the animals. Although aminoacyl-tRNA synthetase activity is unaffected by cold (10°) acclimation, transferase activity shows a 60% increase compared with levels in fish maintained at 21°. This change is similar to the apparent increase in liver protein synthesis in 10°-acclimated fish as measured by the incorporation of radioactive amino acids into protein 30 min. after arterial injection. The findings thus suggest a possible mechanism for control of protein synthesis in cold acclimation; however, it depends on whether the step catalyzed by the transferase is rate-limiting *in vivo*. Increased activity during cold acclimation may result from conversion from an inactive or dissociated form. Analogy to studies in rat liver suggests that the enzyme involved is aminoacyl transferase I.

Studies on aminoacyl-tRNA synthetases have been made in rat and frog liver in relation to the possibility of control of codon recognition during changes in protein synthesis induced by thyroid hormone. Three enzyme fractions capable of activating and transferring phenylalanine to rat liver transfer RNA were separated by ammonium sulfate fractionation and DEAE-cellulose chromatography of rat liver extracts. Activities comparable to two of these fractions were found in tadpole and frog liver extracts. Preliminary studies indicate that two different types of phenylalanine-acceptor tRNA may be charged by these enzymes.

Supported by National Science Foundation grant GB 5194.

Studies with reaction centers from Rhodopseudomonas spheroides. J. R. Bolton and R. K. Clayton.

A reaction-center fraction isolated from *Rhodopseudomonas speroides* chromatophores exhibits light-induced changes in its optical and electron spin-resonance (ESR) spectra. In particular, a bleaching at 870 nm (P870) has been found to be closely correlated with the appearance of an ESR signal with a *g* factor of 2.0023 and a peak-to-peak line width of 10 G. The ESR signal is indistinguishable from light-induced signals found in chromatophores or whole cells.

A careful measurement of the spin concentration showed that the ratio of the light-induced spins to bleached P870 molecules is 1.1 ± 0.1. In addition the formation and decay kinetics are identical within experimental error under a variety of experimental conditions.

Previous work has shown that P870 is a bacteriochlorophyll molecule in a specialized environment and that the bleaching signifies oxidation. The present work provides strong evidence that the product of the bleaching of P870 is the radical cation of bacteriochlorophyll, $P870^+$, and that this radical is the source of the ESR signal in whole cells.

The quantum yield for the bleaching of P870 in reaction centers has been measured, using actinic light of wavelengths 880, 800, 760, and 680 nm. For light absorbed at 880 or 800 nm the efficiency is close to 100%. In a coupled reaction, the oxidation of mammalian cytochrome *c* by $P870^+$ proceeds with nearly the same efficiency.

The above results place definite limits on the possibilities for the identity of the primary acceptor. These possibilities are discussed.

July 23, 1968

The relationship between carbon atom chain length and effectiveness of n-alkylamines in inducing rhythmicity of gastropod smooth muscle. Robert B. Hill.

The isolated radula protractor preparation from *Busycon canaliculatum* has seemed of particular interest because it can be provoked to rhythmicity by the simultaneous presence of two naturally occurring neurohumors, ACh and 5HT. ACh alone will not provoke rhythmicity and 5HT or tryptamine alone do not provoke such regular rhythmicity as they do in the course of relaxing an ACh contracture. A disturbing feature of the induced rhythmicity as a model of natural rhythmicity is the high concentration of indolealkylamines required. However, other biogenic amines are quite ineffective in inducing rhythmicity. N-alkylamines of differing carbon chain length were tested as analogs of the alkylamine side-chain of 5HT and tryptamine. In the series from methylamine to undecylamine, pentylamine and hexylamine were found to be the only substances which consistently and significantly potentiated twitches, and they did so at the 10^{-6} *M* level, which may be compared to potentiation by 5HT and tryptamine at the 10^{-7} *M* level. Pentylamine and hexylamine relaxed acetylcholine contractures, and in so doing provoked rhythmicity, at the level of 3×10^{-6} *M*, whereas 5HT and tryptamine relax with rhythmicity at the level of 10^{-8} *M*. Acting alone, pentylamine and hexylamine induced irregular rhythmicity at the 10^{-6} *M* level while 5HT and tryptamine induced more regular rhythmicity at the 10^{-8} *M* level. Thus the 5 carbon and 6 carbon atom analogs of the alkylamine side chains of 5HT and tryptamine function at concentrations 1000 times lower than the natural neurohumors in inducing rhythmicity of the radula protractor.

Local variations of pigmentary patterns and chromatophoric responses in the fiddler crab, Uca pugilator. K. RANGA RAO AND MILTON FINGERMAN.

Dimorphic pigmentary variants can be distinguished among specimens of *Uca pugilator*. One variant has a dark patch in the metagastric area of the carapace while the other has a light patch. The variant with a dark patch occurs at Levy Bay, King Bay and Porter Island Peninsula, Florida, but occurs neither at Alligator Point, Florida, nor Chapoquoit in Falmouth, Massachusetts. The variant with a light patch occurs at the five localities mentioned above. The chromatophoric responses of both variants obtained from Florida were studied. Both variants exhibited different degrees of adaptation on black and white backgrounds. These responses varied with the size of the crabs and the area from which they were collected. Among eyestalkless individuals the melanin was maximally concentrated in the chromatophores of both variants. However, the degree of white pigment dispersion in the eyestalkless crabs varied with the size of crabs and the area from which they were collected. The red pigment was nearly maximally concentrated in the chromatophores of eyestalkless individuals of the dark patch variant, whereas it was in an intermediate state of dispersion in the light patch variant. The melanin in the chromatophores of eyestalkless individuals of the light patch variant dispersed in response to bright light, whereas the melanin in the chromatophores of eyestalkless dark patch crabs did not.

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Reproductive performance of insects from U. S. Biosatellite II. D. S. GROSCH.

No sweeping general cytological conclusion can be made about the effect on cells of radiation delivered during orbital flight. Both cell type studied and criterion of effect are important considerations. Enhanced, null, and antagonistic effects have been demonstrated in *Tribolium, Drosophila* and *Habrobracon* and all three kinds of effects can be demonstrated for cells of the ovary.

JULY 30, 1968

Formation of the first cleavage furrow in a telolecithal egg (Loligo pealii). JOHN M. ARNOLD.

First cleavage in the squid egg is meroblastic with the furrow appearing in the center of the blastodisc and proceeding toward the edges. Therefore, it is possible to obtain a temporal sequence of furrow formation by progressively taking sections from the blastodisc edge toward the center.

Electron micrographs show the earliest indications of the furrow as a distal flattened region, with longitudinal surface folds running toward the furrow. Immediately below this is a dense layer of fibrils approximately 70 Å in diameter which run parallel toward and below the base of the furrow. These fibrils appear to be derived from membrane-bound dense bodies which originate in the Golgi apparatus. The folds contain many vesicles and tubules which form an irregular tubular network. The older furrow extends downward into the cytoplasm but still has the dense fibrillar region at its base and an average of 17 longitudinal folds in an expanded region at the furrow base. Frequently associated with the furrow base are large multivesiculate bodies which occasionally open and empty vesicles toward the furrow. Below and around the furrow base there is an extensive area of smooth endoplasmic reticulum-like vesicles which are oriented toward the furrow. When cleavage is completed the base of the furrow is in contact with the yolk mass and only a few longitudinal folds remain, but the dense fibrillar layer is still evident.

A cleavage mechanism can be postulated in which the fibrils are associated into a contractile band which is linked to the plasma membrane. This band forms at its ends, is anchored at the edge of the blastodisc, and upon contraction cuts through the cystoplasm. As contraction occurs the fibrils are laterally compressed together by the resistance of the cytoplasm. This causes the longitudinal folds to form. Then the band is bent back upon itself. As the furrow divides the cytoplasm, most of the longitudinal folds unfold and contribute their plasma membrane to the "new" surface of the blastomeres. In addition, new membranes must be added, possibly from either the smooth endoplasmic reticulum-like vesicles or from the tubular network in the folds.

This work was supported by NIH Grant HD 02688.

On the distribution of genetic variation in nature. ROGER MILKMAN.

The data on allelic variation in nature, and particularly the many recent studies of electrophoretic mobility variants of numerous enzymes, suggest a generalization comprising four parts: (1) At most gene loci in any species there are between two and five alleles that attain significant frequencies (over 1%) anywhere. (2) The achievement and maintenance of these frequencies is due to (single-locus) heterosis. (3) There are relatively few locally-adapted alleles; the common demands of membership in the species outweigh the special demands of a particular external environment within its range. (4) Certain alleles are fixed in local populations from time to time due to the limitations on the number of polymorphisms that can be maintained in finite populations. Thus distant populations of a species differ mainly in their samples of genes from a common species library.

The reunion of alleles with a history of heterosis can result in the production of some superior progeny by parents of distant origins. This phenomenon is frequent; it would not be expected if local adaptations were the preponderant determinants of genotypes.

Numerous counter-examples suggest that this generalization has many exceptions at every level from locus to species. Yet it may be a useful starting point.

The accurate estimation of the number of loci in a given species is now of fundamental importance; the vast difference in estimates generated by different methods must be resolved.

Rapidly contracting muscles used in sound production by a katydid. R. K. JOSEPHSON AND H. Y. ELDER.

The song of the katydid, *Neoconocephalus robustus,* consists of a series of chirps, each produced by a stroke of the forewings against one another, but the frequency of the wing movements and chirps is so high (150–200 per second) that to a human listener the song is a loud, continuous buzz. Wing movements at frequencies exceeding 100 per second are known for a number of insects, but such frequencies have hitherto always been associated with muscle which is termed asynchronous because there is not a 1:1 relation between muscle action potentials and contractions. Recordings from extra-cellular electrodes implanted in the singing muscles of *Neoconocephalus* reveal that there is a muscle action potential associated with each contraction; these muscles are, therefore, of the synchronous type. The contraction-relaxation time of these muscles during singing is approximately 5 msec., making them among the fastest synchronous muscles in the animal kingdom. The myofibrils of the singing muscles are thin sheets which radiate from the center of the muscle fiber. The myofibrils are densely invested with sarcoplasmic reticulum, a characteristic of very fast synchronous muscles. Mitochondria comprise approximately 44% of the volume of these muscles.

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AUGUST 6, 1968

Shark and dogfish studies: Pulsatile pressure extracorporeal blood oxygenation by means of silicone membrane "heart-lung" devices and artificial kidney. C. LLOYD CLAFF, ARMAND A. CRESCENZI AND ARNOLD J. LANDÉ.

After reading the title: Three questions may naturally come to mind: Question number one: Why use sharks and dogfish instead of dogs? Question number two: Why a *membrane* oxygenator? Question number three: Why pulsatile pressure? Shark blood retains 2–2½% urea, while other vertebrate blood retains only 0.01–0.03% urea. The shark therefore is especially suitable for evaluation of artificial kidney devices and oxygenating devices.

Why a membrane oxygenator? There is not a case where, in a biological system, blood is in direct contact with a gas, without a membrane at interface.

Why pulsatile pressure? Pulsatile pressure insures a new interface at every pulse—hence a better oxygenation per square meter of surface. Clinical evidence now shows steady pressure causes areas of anoxia, with resulting brain damage, and kidney complications. A nurse shark 4½ feet long was put on "bypass" at Florida Oceanographic Society Marine Station, Stuart, Florida, using Dr. Landé's artificial gill and the Claff-Crescenzi pulsatile pressure method of oxygenation, except fluoro-carbon was used instead of oxygen, and the carbon dioxide was washed out with filtered sea water. The extracorporeal circuit was a cannulation from the caudal vein, through a DeBakey pump, thence to a membrane artificial kidney, to a membrane heart-lung device. The oxygenated blood was returned to the body by cannula in the conus arteriosus. Venous return was assured by implanting a cannula in the heart cavity, sewing up the cavity, and connecting the cannula to a source of pulsating negative pressure.

Anesthesia was accomplished with tricaine methane sulphonate; curare 3 mg./kg. every 30 minutes; heparin 3 mg./kg. intravenous. Results: Hematocrit arterial, 30; venous, 33; pH arterial, 737; venous, 7.31; pCO_2 arterial, 17; venous, 22; pO_2 arterial, 350; venous, 180. Blood urea 428–420 net change of 8; no blood sugar present.

The assistance of Louis Sonstegard, Katherine McCusker and Marianne Broome is gratefully acknowledged.

Asexual and sexual reproduction of Pygospio elegans (Polychaeta) in Barnstable Harbor, Massachusetts. KATHERINE D. HOBSON AND ROGER H. GREEN.

We observed the cycle of asexual and sexual reproduction of *Pygospio elegans* Claparède during a study (1967 to 1968) of the intertidal fauna near Huckins Island. Every month, eight samples of the fauna retained by a screen of 1 mm. mesh were taken from each of two stations (60 cm. and 90 cm. above mean low water).

Pygospio elegans reproduces asexually by breaking into fragments, usually of two or three segments, each of which then regenerates a head and tail. Fragmentation was most intense (increasing the population by a factor of 10) during April and May of both years at the lower station. By June or July, the population had returned to the original level. At the upper station, intense fragmentation occurred only in the spring of 1968.

Females with egg capsules were observed from March through May, but comprised less than 7% of the pre-fragmentation population. Because at least 30% of the water in Barnstable Harbor is exchanged on each tidal cycle, a female releasing 500 larvae, which have a pelagic life of two weeks, would be replaced by only 0.02 larvae. Thus this population of *P. elegans* must be maintained by sexual reproduction of adults outside the harbor or by asexual fragmentation. The fragments would not be subject to tidal flushing because they remain in the tube of the adult during the regeneration process.

During spring in Barnstable Harbor, a large portion of the population reproduces asexually and a small portion reproduces sexually. We do not know the role of asexual reproduction in the life history of *P. elegans,* nor the extent of its importance in maintaining the population in Barnstable Harbor.

This research was supported by a grant from the Whitehall Foundation to the MBL Systematics-Ecology Program; SEP Contribution No. 163.

Limb regeneration in adult amphibians. MARC H. DRESDEN.

Investigations during the past 30 years have shown that regeneration of amputated limbs in adult amphibians requires an adequate nerve supply. In an effort to gain a more basic insight into the role of nerve in regeneration, a study of the macromolecular syntheses of the regenerating blastema was initiated.

Both forelimbs of adult newts (*Triturus viridescens*) were amputated proximal to the elbow and regeneration was allowed to proceed to the palette stage (3–4 weeks post-amputation). These animals were then denervated unilaterally (the other arm serving as a control) by resection of spinal nerves 3, 4, and 5. After various periods of time the animals were injected intraperitoneally with a mixture of ^{3}H-thymidine and either ^{14}C-leucine or ^{14}C-uridine. Four to five hours later the animals were sacrificed and the radioactivity incorporated by the blastema was assayed. Within seven hours the denervated blastema begins to show a decreased capacity for uridine incorporation, followed within 25 hours by a decreased capacity for thymidine and leucine incorporation. Sham-operated animals show no change in thymidine and leucine incorporation. Thymidine incorporation decreases to about 25% of the control regenerating capacity, which is equivalent to that found in normal arms and in the proximal regions of regenerating arms. No significant effect of denervation on the incorporation of thymidine and leucine is seen in non-regenerating limb tissues.

In addition, it was found that the changes in thymidine incorporation following denervation can be mimicked *in vitro*. When the blastema derived from animals denervated 48 hours previously are incubated in a tissue culture medium containing ^{3}H-thymidine they incorporate about 35% as much of the isotope as do the blastema derived from undenervated animals or from animals denervated 17 hours previously (at which time thymidine incorporation has not yet begun the decrease *in vivo*).

The changes in incorporation of precursors of RNA, DNA, and protein following denervation precede any morphological changes. The magnitude of the denervation effect and its specificity for regenerating limb tissues indicates that there may be a dual control over macromolecular syntheses in amphibian tissues.

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AUGUST 13, 1968

Segregation of filamentous protein structures from the axoplasm of the squid giant nerve fiber by uranyl ions. J. METUZALS.

Certain heavy metal ions show specificity for reactive groups of protein molecules. The proper choice of metals can be very useful in fractionation, crystallization of desired proteins and in labeling of active sites of proteins by virtue of such specificity. UO_2^{++} ions of a 1% uranyl acetate solution at pH 4.4 display a specific affinity to filamentous protein structures of the squid giant nerve fiber axoplasm. At pH 4.4 the uranyl ions segregate the filamentous elements of the axoplasm in a coiled, threadlike preparation surrounded by a flocculent precipitate. The uranyl ions combine probably with the carboxyl groups of the main amino acids of the protein—the glutamic and the aspartic acids. Segregation cannot be observed at pH 7.8. Affinity to the filamentous axoplasmic protein structures is displayed also by Ag ions, but not by ions of La, Co, Pb and Zn salts.

Because of the fixing effect of the uranyl ions at the macro-molecular level and the simultaneous increase of contrast, the preparations obtained by the uranyl procedure are well suited for an integrated approach using both high resolution electron microscopy and physicochemical methods. Three hierarchic levels of coiled configurations can be observed as a characteristic structural pattern of the thread preparation: coiling at (1) the light microscopic level visualized by interference optics, (2) the fine structural, and (3) the macromolecular levels disclosed by electron microscopy.

Analysis data of the axoplasmic thread, segregated by uranyl ions, show the following composition: N 10.8%; U 18.8%; P 4.6%; Ca 0.6%; Mg 0.08%; 1–2% ribonucleotides. Amino acid composition expressed as moles per cent: Ala 9.0; Arg 3.5; *Asp 11.0;* Cys 0.8; *Glu 14.4;* Gly 10.8; His 1.5; Ileu 2.5; Leu 6.8; Lys 6.4; Met 2.5; Phe 3.5; Pro 5.7; Ser 9.6; Thr 5.7; Tyr 2.5; Val 3.9.

Supported by the Medical Research Council of Canada.

Observations of living giant nerve fibers of the squid by differential interference-contrast microscopy. J. METUZALS, C. S. IZZARD AND M. GOSPODNETIĆ.

Using the differential interference-contrast microscope (Carl Zeiss), one can obtain thin "optical sections" of the living giant nerve fiber of the squid without interference by structures above or below and approach the limit of resolution. For observations with transmitted light, the stellate ganglion with a 5-cm. length of giant nerve fiber, ligated at the distal end, was mounted in sea water. *In situ* giant nerve fibers were observed using the reflected interference-contrast microscope. One cm. of fiber was cleared of surrounding tissue and a fragment of aluminized coverslip, as a reflecting surface, was inserted between the fiber and the underlying mantle wall.

Parallel threadlike elements of 1–2 μ diameter and irregular dense bodies of varying size represent the main structural features of the axoplasm at the light microscope level. A peripheral ectoplasm and a central endoplasm can be distinguished by differences in packing and arrangement of these elements. The ectoplasm exhibits a dense parallel packing of straight threadlike elements, whereas in the endoplasm the threads often show a looser reticular array. In the ectoplasm the threadlike elements are oriented at an angle of 5–15° to the longitudinal axis of the fiber. In optical sections of the upper and the lower ectoplasmic regions, the direction of this angle is reversed with respect to the axis of the fiber, indicating that the threads are wound helically along the fiber. In certain locations of the endoplasm, sinuous threads are in register across the width of the fiber resulting in the overall appearance of large scale cross banding. The structural configurations observed in the living fibers correspond well to those observed by light microscopy of stained, thick plastic sections of material prepared for electron microscopy.

Supported by MRC of Canada and NIH grants.

Quantitative radioautography of vitellogenesis in Artemia salina. REV. J. D. CASSIDY, O. P., C. F. STARMER AND L. J. BEAUREGARD.

Genetic transcription sites were localized by grain frequencies counted on series of 0.5-μ cellular sections in differentiating ova and accessory cells of adult brine shrimp. Isotope distribution was scored after *in vivo* exposure to tritiated thymidine, uridine and actinomycin-D. Nine incorporation sites, dosage and rate of isotopic uptake, as functions of time, were tested using a multivariate general linear model in the IBM 1130 computer. The primary model related multiple sites in the vitellarium to time effect and precursor effect as a 3×3 multivariate factorial design. Hypotheses tested specific incorporation of precursors at a particular time and similarities between precursors over time and time averaged over precursors. Results were evaluated using the multivariate likelihood ratio to determine treatment effects (time and precursor) on the entire multivariable observation (incorporation sites) and individual "F" ratios (adjusted according to the Bonferonni inequality) to determine treatment effects on individual incorporation sites.

Thymidine, uridine and actinomycin-D were incorporated in similar amounts, pooled over time, by the oocyte yolk platelets and nurse cell nuclei. Comparison of thymidine and uridine indicated comparable isotopic uptake by nuclei of the follicular layer and lower ratios within the nurse cell cytoplasm and the oocyte-nurse cell interface. Evaluating time effect on precursor incorporation yielded no difference in uptake from 5 to 10 and 5 to 20 min. in follicular cytoplasm and the region adjacent to yolk platelets. Tests for dissimilar incorporation of each precursor/time determined no interaction at the oocyte-nurse cell interface, follicle cytoplasm and yolk platelets; significant interaction adjacent to yolk platelets and follicular nuclei; and highly significant interaction involving the germinal vesicle, cortical ooplasm, nurse cell nuclei and cytoplasm. This cytological-cytochemical-computerized approach identified yolk platelets as a cytoplasmic center of informational template synthesis in the developing egg chamber.

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GENERAL SCIENTIFIC MEETINGS

AUGUST 19–22, 1968

Abstracts in this section are arranged alphabetically by authors. Author and subject references will be found also in the regular volume index, appearing in the December issue.

An analysis of cleavage furrow formation in the egg of Loligo pealii. JOHN M. ARNOLD.

Cleavage furrows in the meroblastic squid egg form from the center outward. The base of these furrows is associated with many 70 Å fibrils which arise from Golgi-derived, membrane-bound dense bodies. In an earlier report a theory of fibrillar contraction in furrow formation was put forth. Simply stated this theory involves a progressively distal formation of a fibrillar band below and associated with the plasma membrane. A cleavage furrow is formed when this band contracts. The experiments described below test this hypothesis.

If fibrillar contraction cuts into the cytoplasm to form the furrow, then curvature of the egg surface is necessary. Prior to first cleavage the blastodisc was flattened with a coverslip. This not only prevented furrow formation, but also stopped it once it had begun. Release of the pressure resulted in furrow formation a few minutes later. Heavy compression before cleavage prevented furrowing and resulted in a delayed, irregular cleavage pattern. Flattening one side of the blastodisc parallel to the first furrow caused unilateral delay or complete inhibition. Lateral flattening changed the shape of the blastodisc so the future ends of the first furrow were well below the upper margin of the yolk mass. When the furrow formed it indented the yolk. In normal eggs third cleavage is asynchronous with the anterior furrows forming slightly before the posterior furrows. These furrows appeared attached to the second furrow because it is distorted just at the junction with the forming third furrow. This strongly suggests the forming furrow is contracting. By using an ultraviolet microbeam it was possible to irradiate the forming fibrillar band and cause the second furrow to disappear unilaterally. A very faint birefringence, possibly the fibrillar band, is apparent in the future furrow region just prior to actual cleavage. All of these results and observations are consistent with a mechanism of furrowing by a contractile fibrillar band.

This work was supported by NIH Grant HD 02688.

A ciliary activity inhibitor extracted from the nidamental gland of Loligo pealei. BURR G. ATKINSON AND N. A. GRANHOLM.

An extract prepared from the nidamental gland of the squid causes adjacent cilia to adhere to one another, but the effect is *not* a general clumping: groups of cilia appear attached at their tips. The overall effect of the factor is to bring the swimming movements of ciliated organisms to a halt by reducing the *effectiveness* of ciliary activity; the actual beating continues.

Histochemical examination of the nidamental gland discloses that it is primarily composed of columnar cells containing a substance which stains metachromatically with toluidine blue and which is PAS and alcian blue (pH 2.5) positive. While the squid jelly case and dry smears of the crude viscous extract exhibited similar histochemical properties, the accessory nidamental gland did not. In keeping with this histological evidence, biological activity could not be extracted from the accessory nidamental gland, but was obtained from the nidamental gland and the egg jelly case.

The viscous preparation initially extracted from the gland has a milky-white color. Low-speed centrifugation produces a clear supernatant fraction and a viscous milky-white pellet. Although the clear supernatant was used as the source of biological activity, more activity could be extracted from the pellet by homogenization and centrifugation. The pooled supernatants were centrifuged at 105,000 × g and the activity was recovered in the supernatant fractions. The ratio of proteins to neutral hexoses in these final supernatant fractions was 5:1. The biological activity of this fraction was not labile to dialysis against Versene or 0.1 N NaCl, nor to 10 minutes in a boiling water bath, The high-speed extract could be prepared in Millipore-filtered sea water, distilled water or CMF sea water; it could then be lyophilized and resuspended in the desired medium. Further purification of the lyophilized fraction involved a separation of the biological activity from most of the proteins on a G-25 Sephadex column.

Supported by Grant 5-TO1-HD0014–09 from the Public Health Service.

Cellular clots in Pagurus longicarpus and the possible role of microtubules. F. B. Bang.

Clotting of amebocytes was studied following the cutting of the antenna and placing the cut end in sea water. N-ethyl maleimide in concentrations as low as 0.005 mM in sea water was absorbed over a period of hours by *Pagurus* and acted as an anti-cellular anticoagulant. The effect persisted for several days. Most of the animals exposed to this concentration survived. Electron microscopy of clotted cells, following glutaraldehyde fixation and later treatment with osmium, showed numerous microtubules which apparently strengthened the second stage of clotting. These were not found one and two minutes after cutting the antenna but were found from three minutes to 11 days afterward. Several anaesthetics and drugs known to affect the microtubules of spindle figures during mitosis altered the clot of *Pagurus* when the cells were allowed to spill into a drop of an appropriate solution. These presumably acted by keeping the cells from entering into the second stage of the clot. Urethane (6%) and hexylene glycol were the most effective. The interrelationship of these findings is as yet unexplored.

Electron microscopic examination of subcellular components containing melanophorotropic activity, separated by density gradient centrifugation, from neurosecretory cells in the eyestalks of the fiddler crab, Uca pugilator. Clelmer K. Bartell, Kenneth May and Milton Fingerman.

The melanin-dispersing hormone from the eyestalks of *Uca pugilator* is thought to be stored within neurosecretory granules of the sinus gland. In an effort to isolate these granules, the microsomal fractions of eyestalk extracts were prepared by differential centrifugation of tissue which had been sonicated in 0.82 M sucrose. Much of the melanin-dispersing activity of the extract was contained in the microsomal fraction. The microsomal pellet, resuspended in 1.00 M sucrose, was layered over a discontinuous sucrose gradient, from 1.00 to 1.75 M concentration, and centrifuged for two hours at $200{,}000 \times g$. Bio-assay of the fractionated gradient indicated the presence of two distinct fractions having different densities. The fractions were adjusted to a concentration of 0.82 M sucrose, aliquots were assayed for melanin-dispersing activity in eyestalkless *Uca,* and the remainder of each fraction was reduced to a pellet by centrifugation. The pellets were then prepared for electron microscopic examination. The less dense fraction contained an assortment of electron-lucent, vesicular membranes and an occasional neurosecretory granule. The more dense fraction was a non-homogeneous mixture of cell fragments including numerous electron-dense particles having a size and electron-density similar to the neurosecretory granules observed in sections of the intact sinus gland. In contrast, extracts prepared in physiological saline, instead of sucrose, contained only the less dense active fraction: the granules appeared to be disrupted by extraction in saline.

These results indicated that much of the hormonal activity can be isolated in association with specific particulate fractions from the disrupted cells of the eyestalks from *Uca.* The activity is probably contained within neurosecretory granules and fragments of the granules. Further refinement of the technique should produce fairly pure preparations of granules that will be useful for chemical analysis.

Supported by Grant GB-7595X from the NSF.

Morphology of the eye of the flame fringe clam, Lima scabra. Allen L. Bell and George J. Mpitsos.

The electrophysiology of the vision of *Lima* has been studied by one of us (Mpitsos). The eyes give both an on-response and an off-response to stimulation with light. This report gives the preliminary results of fine structure studies which were undertaken with the purpose of locating morphological correlates to the electrophysiological data.

The eyes of *Lima* are cup-shaped organs each about 600 microns long, 200 microns wide and 150 microns deep, lying along the edge of the mantle. Longitudinal sections through the cup show that the retina consists of a pseudostratified epithelium comprised of two cell types: visual cells and pigment cells.

The visual cells have a large inner segment or cell body attached by a short stalk to an outer segment or rhabdome. These cells are 40 to 50 microns long, 10 to 15 microns wide and stand vertically on a thick basement membrane. In addition to a nucleus, mitochondria and Golgi material, the base of the visual cell is filled with a large amount of membranous material consisting of tubules and vesicles. Visual cell outer segments measure 8 to 10 microns in diameter and have numerous microvilli extending outward from their surface interdigitating with those of adjacent outer segments. Pigment cells lie between the visual cells. Their nuclei are located apically and the cells narrow as they approach the basement membrane. The apical surface of the pigment cells is at the level of the stalk of the visual cells. Thus the outer photoreceptor portion of the visual cells extends beyond the most apical part of the pigment cells.

Nerve fibers are found between the basal surface of the retinal cells and the basement membrane. Occasionally one of these fibers appears to end in a swollen bulb which contains clear vesicles 600 to 800 Å in diameter and dense cored granules 1200 to 1500 Å in diameter.

Preliminary examination of the "lens" of the eye reveals large bundles of cilia and numerous processes which appear to be neural. Nerve terminations are numerous in this region. The role, if any, played by the lens structure in the visual response is being examined.

Part of this research was supported by a Grass Foundation Fellowship, 1967, to George J. Mpitsos.

Electrical coupling of embryonic cells by way of extracellular space and specialized junctions. M. V. L. BENNETT AND J. P. TRINKAUS.

In cleavage stages of the egg of the teleost, *Fundulus heteroclitus,* current applied in one cell produces very nearly the same potential in all cells. In later stages of blastula formation, current in one cell produces a larger potential in that cell than than in distant cells. However, as distance from the polarized cell is increased, the potential rapidly approaches that in the distant cells. If current is applied in the segmentation cavity of the gastrula, the same potential is recorded throughout the cavity and in all cells as well. The resistance between the cavity and the exterior is very high indicating that junctions connecting outer margins of the superficial cells (Trinkaus and Lentz, *J. Cell. Biol.,* 32: 139, 1967) seal off the cavity from the exterior and are functionally "tight." Also, the resistance of the outer membrane of superficial cells is high (*ca.* 100 kΩcm.^{2}), much higher than that of underlying membrane abutting on the segmentation cavity. Thus, current can pass from cell to segmentation cavity to cell with little leak to the exterior, and cells can be coupled by way of intra-embryonic extracellular space. Superficial cells also appear to be coupled by way of junctions between them because cells near a polarized cell can be at a higher potential than the underlying segmentation cavity. Coupling of embryonic cells by way of junctions is also indicated since pairs of blastomeres isolated mechanically are usually coupled and single cells reassociated *in vitro* can develop coupling. In this embryo, junctions can transmit small molecules from cell to cell over short distances, but transmission over large distances must be primarily by way of extracellular space.

Dogfish white blood cells: protein synthesis in vivo and in vitro. GRACIELA C. CANDELAS, SANFORD TEMES, JOSÉ R. ORTIZ AND SEYMOUR GELFANT.

The technique for culturing dogfish blood cells has been developed in our laboratory. Studies have indicated that the metabolism of cells in culture may be different than *in vivo.* We have studied protein synthesis of dogfish white blood cells *in vivo* and *in vitro* under a number of conditions by following the incorporation of tritiated amino acids in TCA-precipitable protein. Preliminary studies of the protein synthesizing units of these cells were also undertaken.

A comparison of the rates of protein synthesis *in vivo* and in culture medium (20% plasma, 80% elasmobranch Ringer's) revealed a sharp drop when the cells were placed in culture. The rate of protein synthesis was reduced by about 65% *in vitro.* It was found that this drop occurs within the first few minutes of culture and that the rate remains reduced throughout the first hour. However, by 24 hours of culture, the rate of protein synthesis returns to almost 100% of the *in vivo* rate.

If the cells were cultured in 100% plasma, the drop in protein synthesis was minimized. After 72 hours of culture, cells in 100% plasma displayed higher rates of protein synthesis than cells in the usual medium (20% plasma). Thus plasma was shown to be beneficial either in promoting proliferation, activating protein synthesis, or both.

Density gradient analysis of the 7000 × *g* supernatant revealed some protein synthesizing activity in a heavy peak, but most activity was found in the light monosome peak, indicating the need for an RNase inhibitor in these studies. Analysis of the resuspended 105,000 × *g* pellet yielded a heavy peak containing all the activity. The activity in this heavy region was insensitive to standard RNase and deoxycholate treatments.

Supported by research grant GB-2803 from the National Science Foundation.

The use of luminescent systems and image intensification as cytochemical probes. I. The distribution of ATP in the muscle cell. G. B. CALLEJA, GEO. T. REYNOLDS AND R. J. PODOLSKY.

Because of its sensitivity to photons, the image intensifier may be used to detect the spatial distribution of photon sources not discernible by the unaided eye. The localization of microsources of light by image intensification has already been achieved in *Noctiluca,* a bioluminescent dinoflagellate. In addition, cells which are not luminescent may be made to emit light if a luminescent system is introduced into them. If the system introduced is complete except for one necessary component, not only the amount, but also the spatial distribution of this one component can be determined. Luminescent systems which possess potential cytochemical uses include firefly luciferin-luciferase for the detection of ATP, aequorin from *Aequorea* for Ca^{++}, bacterial luciferase for elements of the electron flow system, etc.

We report in this paper the preliminary results of our studies on the distribution of ATP in the muscle cell. Single fibers were isolated from the semitendinosus muscle of *Rana pipiens.* Firefly luciferin-luciferase was introduced as a microdrop onto the surface of fibers with or without sarcolemma. The preparations, viewed through a microscope attached to an image intensifier, were then photographed in the dark. We found that most of the ATP that resulted in light emission was not preferentially associated with any structure identifiable by means of the light microscope. To the limits of resolution so far achieved (the order of 1 micron), it appears that ATP is distributed uniformly along the longitudinal axis of the cell. In the intact fiber, luminescence was restricted to the cell surface. Thus, whereas the sarcolemma effectively excluded luciferase from the sarcoplasm, it did not prevent contact between ATP and the enzyme. The kinetics of emission suggests that light from the intact fiber was due to ATP either leaking out or being regenerated on the cell surface.

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Effect of cycloheximide on ciliogenesis, ciliary regeneration, and protein synthesis in Arbacia embryos. FRANK M. CHILD AND MATTHEW N. APTER.

Regeneration of amputated cilia in protozoa generally requires some protein synthesis, although assembly of regenerating cilia can occur in the absence of protein synthesis if the necessary proteins have been allowed to accumulate. In *Paracentrotus,* Auclair and Siegel, 1966 (*Science,* 154) conclude that protein synthesis is not required for ciliary regeneration, since puromycin will not prevent regeneration. They suggest that all of the required proteins exist in a pre-formed pool drawn upon during cilia formation. Do sea urchins differ from protozoa in the control mechanism governing the formation of cilia?

We have now shown in *Arbacia* that CH (cycloheximide, 5 mg./ml.) will significantly delay the formation of motile cilia in regenerating blastulae and early gastrulae. CH will also delay the normal formation of cilia in blastulae when CH is applied after fertilization as late as the elapse of 84% of the scheduled time of ciliogenesis.

Cycloheximide inhibits the incorporation of C^{14}-valine into TCA-insoluble protein of late blastulae and early gastrulae. Five mg./ml. of CH permits incorporation at 10% of the control rate; lower concentrations of CH inhibit less.

Since we assay the formation of cilia by sensitively detecting the first appearance of motile shafts, the delays produced by CH suggest that ciliogenesis and ciliary regeneration in

Arbacia are processes directly dependent on the synthesis of at least one protein in the half-hour or so preceding the formation of motile shafts. Inhibition of protein synthesis by CH could act by delaying the accumulation of this protein required for shaft assembly.

This work was supported by NIH grant GM-13993.

Immotility and motility of acoel turbellarian spermatozoa, with special reference to Polychoerus carmelensis. DONALD P. COSTELLO AND HELEN M. COSTELLO.

Examination of living adult *Polychoerus carmelensis* over an extended period of time (10 weeks), from beginning to height of their breeding season, has yielded information on spermatogenesis, spermiogenesis, oogenesis, and on activities in such organs as the bursa seminalis. Since *Polychoerus* can be effectively flattened, it was possible to study living specimens under phase oil immersion objectives, and to take photomicrographs of salient features.

The bi-axial filamented spermatozoan is almost invariably immotile, as seen in the distributed testes, sperm ducts, ejaculatory ducts, or when emitted into sea water. These observations are in perfect accord with the known fact that although fertilization of the eggs by the sperm of the same animal would be anatomically possible in the hermaphroditic Turbellaria, self-fertilization is unknown or very rare. Mating occurs by reciprocal copulation, and the sperm of another individual are introduced by way of the female antrum into the bursa seminalis (seminal receptacle). In this bursa, and in the paths and cavities leading from it to the mouthpieces (nozzles), the spermatozoa become intensely active, wriggling with snake-like undulations. The motion is caused by two lateral undulating membranes on opposite sides of the long flattened body of the spermatozoan. The wave motion in these begins at the posterior end and progresses forward.

We have observed active spermatozoa proximal to (nearest the bursa) the mouthpieces, where some appear to be attempting to enter the tiny tri-radiate cuticular canals. Inert, dark thread-like material is frequently seen at the distal ends of the mouthpieces. No evidence was obtained indicating that these threads are active, living sperm. A few active sperm were observed, however, passing between or around the mouthpieces. This suggests that the function of the mouthpieces is not necessarily to direct the sperm toward the eggs. They may have the function of hindering and digesting excess sperm.

Aided by a grant from the National Institutes of Health, GM-15311.

Oxygen-linked dissociation of hemocyanin. I. Experimental evidence. HENRY A. DEPHILLIPS, JR. AND K. E. VAN HOLDE.

The investigation of the oxygen binding properties of hemocyanin obtained from the squid, *Loligo pealei,* has shown a novel dependence of per cent oxygenation on molecular size. Confining our attention to a concentration of 5 mg. protein/ml., an ionic strength of 0.125 (0.1 ionic strength buffer plus 0.01 M Mg^{++}) and a temperature of 20° C. we find that over the pH range of 6.0 to 8.3 in the absence of oxygen (under argon) the 59S component predominates (about 95% 59S species and 5% of non-oxygen binding protein). In the same pH range for samples equilibrated with oxygen in air the 59S component predominates below pH 7.1 and above pH 8.0. Between these values, however, the amount of 59S decreases as the pH increases, to a minimum at pH 7.5, where the 19S component is the only molecular species present, and then increases to 95% at pH 8.0 once again. A similar phenomenon is observed when samples are equilibrated under pure oxygen. In this case, the 59S component is stable below pH 6.8 and above pH 7.4. The decrease in the 59S species reaches a maximum at pH 7.1 where the 19S component is the only molecular species present.

That these are true equilibrium phenomena was demonstrated in an experiment in which the relative amounts of 59S and 19S components were varied by varying only the amount of oxygen in equilibrium with the solution.

Correlation of all these results is achieved if the amount of 59S component is displayed as a function of percent oxygenation. This graph shows that this component predominates below 50% saturation and above 95% saturation. Above 50% saturation, dissociation to the 19S component occurs and is complete in the range of 75 to 90 per cent saturation.

This research was supported by Public Health Service Grants HE-11671 and HE-12326.

Excitation and inhibition of Purkyně cells in the cerebellum of Mustelus canis. John C. Eccles, Helena Táboříková and Nakaakira Tsukahara.

The selachian cerebellum resembles histologically cerebella of higher vertebrates. However, basket cells are absent, though in the molecular layer there are numerous stellate cells that may be regarded as undeveloped basket cells. In investigating the mode of action of the various cells, we utilized the same techniques as for the mammalian cerebellum. After immobilization by intramuscular injection of Tubocurarine, the spiracles are perfused by aerated sea water, the cerebellum is exposed and the skull is rigidly clamped. Concentric needle electrodes are employed for stimulating with currents driven by 0.2-msec. pulses of 10 to 90 volts. One stimulating electrode in the stratum fibrosum excites nerve fibers passing to cerebellar cortex, the mossy and climbing fibers, and also Purkyně axons. A surface electrode stimulates parallel fibers passing laterally from the granule cells. Glass micropipettes (3 M NaCl, 2 to 5 MΩ) and (3 M KCl, 10 MΩ) record, respectively, field potentials and intracellular potentials from Purkyně cells.

Stimulation of Purkyně axons gives field and intracellular potentials. Axonal spike potentials precede slower spike potentials of cell invasion. The depth profile suggests that antidromic invasion fails in superficial dendrites. With double stimulation there is recovery, often to low supernormality around 50 msec. Superimposed on the antidromic response there is often a rhythmic wave that by depth profile and intracellular recording is identified as compounded of all-or-nothing climbing fiber responses, initial repetitive spikes at about 250/sec. being superimposed on a 20-msec. depolarization. Climbing fibers are spontaneously active, often in bursts, phases up to 20/sec., then silences of seconds.

Parallel fiber stimulation depresses antidromic invasion of Purkyně cells, often in two phases. The initial depression of about 50 msec. is largely a sequel of the initial activation of Purkyně cells, but the later depression—from 70 to 500 msec.—gives evidence of prolonged inhibition, presumably produced by the stellate cells, which thus resemble basket cells.

This work was supported by the Grass Foundation.

The response of the red chromatophores of the prawn Palaemonetes vulgaris to cyclic 3′,5′-adenosine monophosphate. Milton Fingerman, Robert D. Hammond and Renate Schlenz True.

Cyclic 3′,5′-adenosine monophosphate has been proposed as the intracellular mediator of the action of several hormones including intermedin. The following experiments were devised to determine a possible role of cyclic 3′,5′-AMP in mediating the actions of the pigment-dispersing and pigment-concentrating hormones on the red chromatophores of *Palaemonetes vulgaris.*

Solutions of cyclic 3′,5′-AMP (0.15–15.00 mM) prepared in isosmotic sea water were tested *in vitro* on the red chromatophores of the epidermis adhering to the exoskeleton dorsal to the heart. The section of exoskeleton with adhering epidermis was cut into two portions, one serving as the control for the other. In proportion to the concentration in the solution, cyclic 3′,5′-AMP dispersed the red pigment in chromatophores whose pigment was maximally concentrated at the outset, but had no red pigment-concentrating action on chromatophores with maximally dispersed pigment. The pigment-dispersing response to 3.75 mM cyclic 3′,5′-AMP was independent of the calcium concentration (0.0–4.6 grams per liter) in isosmotic solutions. In contrast, it was previously shown that the response of these chromatophores to a constant amount of red pigment-dispersing hormone (RPDH) is directly related to the calcium ion concentration in the medium and that of all the substances tested, calcium ion permitted a response to RPDH at least twice as large as that permitted by any other substance.

These results suggest the following hypothesis of the action of RPDH. RPDH causes a rise in the calcium concentration within the chromatophore. An increase in the internal calcium concentration is the signal that activates adenyl cyclase which catalyzes the synthesis of cyclic 3′,5′-AMP. The amount synthesized is proportional to the amount of RPDH and to the amount of calcium that entered the chromatophore. Cyclic 3′,5′-AMP then triggers pigment dispersion in proportion to the quantity that was synthesized.

Supported by Grant GB-7595X from the NSF.

A new and improved source of trehalase. FRANK M. FISHER, JR. AND ROBERT O. MCALISTER.

The enzyme trehalase (α-α-1-glucoside 1-glucohydrolase, EC 3.2.1.28) has been isolated from a bacterial symbiont from the midgut of *Blaberus craniifer,* a tropical cockroach. The enzyme has been purified 80-fold and some of its physical characteristics determined.

The bacteria, a species of *Aerobacter,* were grown in minimal salt media fortified with 5% trehalose (α-D-glucopyranosyl 1-glucopyranoside) and the enzyme liberated by alternate freezing and thawing with intermittent sonication. The supernatant was treated with $MnCl_2$ followed by acetone (30% V/V) at −7° C. The acetone precipitate was resuspended in citrate buffer (pH 4.6) and subjected to further purification on a DEAE-cellulose column. Elution of the enzyme was effected by a stepwise gradient of increasing pH followed by increasing concentration of NaCl.

The purified enzyme is specific for trehalose and does not have any activity toward 16 other oligosaccharides, glycogen, starch, or any phosphate esters of glucose. The pH optimum is 4.6 and there is little inhibitory effect of heavy metals. The K_m is 2.24×10^{-3} *M* and the V_{max} is about 290 μM hydrolyzed Mg^{-1} protein $hour^{-1}$. Due to the absence of other carbohydrases in the crude supernatant and the relative ease of purification, this trehalase affords a rapid and easy method of determination of this unique disaccharide.

Supported by Grants (C-239) from the Robert A. Welch Foundation and the USPHS, GM 12263.

The cysteinyldopa-iron pigmentation of red species. PETER FLESCH.

Red hair of man, dog, rabbit and red chicken feathers contains amphoteric yellow-orange pigments. These may be extracted at room temperature with dilute acids or alkalies as orange "protopigments" which, upon heating with acids, are converted to red, indicator-like substances. This color change is due to decarboxylation, as shown by Italian chemists. The pigment consists of protein, ferric ion, and a "chromophore" group. The latter is responsible for the indicator behavior and characteristic absorption spectrum of the pigment. Italian chemists have established that the chromophore group results from the polymerization of cysteinyldopa with dopa. In brilliant experiments they synthesized this grouping *in vitro.* The iron apparently links the chromophore group to the protein. In contrast to the black melanoproteins, this protein contains no cystine or dopa. The proteins obtained from the pigments of different species have remarkably similar compositions.

The cysteinyldopa-iron pigments are apparently widespread in nature. It is not known whether they occur in amphibians or fishes. The presence of these pigments can be established by extracting red epidermal structures with boiling acids, precipitating the pigments at their I.P. (in the neutral range for the red "pheomelanoproteins," around pH 3–4 for the chromophore) and obtaining the pure chromophore by repeated filtration through Sephadex G-25 and cation exchange resin columns. The chromophore may be recognized by its purple color in acids, reversible change to orange on alkalinization and characteristic absorption bands.

Because of their unique properties and easy availability of their protein moieties, these pigments are of considerable importance to geneticists, anthropologists and biochemists. Three groups independently studied their chemical nature; hence the terminology ("trichosiderin," "pyrrotrichol," "pheomelanin," etc.) is confusing and will have to be reconciled at international meetings. Until then the term "red pheomelanoprotein" is suggested for the natural pigment and "cysteinyldopa-polymer" for its chromophore group.

Distribution and dispersion patterns of the dwarf tellin clam, Tellina agilis. WILLIAM H. GILBERT.

Field work for this study was done at Barnstable Harbor, Mass., during July, 1968. Random samples were taken at several stations along a transect from the mainland to Horseshoe Shoals. Samples were sieved through a 0.5-mm. mesh; specimens of *T. agilis* were counted, and shell-lengths were measured. The size-class frequency distribution indicated that 2.5 mm. separated juveniles (young-of-the-year) from adults. The upper boundary of the population distribution is around 0.5 feet above mean low water (MLW), where adult density is approximately 50/m.². Adult densities of around 150/m.² were found down to 2.5 feet below MLW, the deepest area sampled. Juveniles were found concentrated around MLW, between the eel grass and the channel, at densities of up to 830/m².

Juveniles exhibited an aggregated dispersion pattern; adult dispersion was not shown to differ significantly from random.

The following correlation was found between clam size and substrate composition: adults and juveniles of mean lengths 4.6 mm. and 1.8 mm., respectively, were found where the median sand particle diameter was 0.5 mm. with a large variance (degree of sorting, D.S., equal to 1.5). Significantly smaller adults and juveniles (3.5 mm. and 1.5 mm., respectively) were found in the area of juvenile abundance, where median particle size was 0.2 mm. with small variance (D.S. = 0.6). The settling velocity of juvenile tellin clams was found to approximate that of sand 0.2 mm. in diameter.

The above data suggest that hydrodynamic forces may be responsible for the intraspecific size-class zonation observed.

This work was supported by NSF funds and a Woods Hole Scholarship through the University of Massachusetts, and aided considerably by the staff and facilities of the Marine Ecology Course at the Marine Biological Laboratory.

Factors influencing the production of byssus threads in Mytilus edulis. KAREN JANE GLAUS.

Measurements were made of the pull required to remove *Mytilus edulis* from the rock substrate at three sites on Sippewissett Beach and from rock crevices in Sandwich jetty, Cape Cod, Massachusetts. At the beach, an average pull of 1.7 kg. removed animals from a site just above the mean low water mark. One kg. removed animals from a site half way up the shore and 0.5 kg. removed animals from the high shore. The pull required at the jetty was 1.0 kg. There is no significant difference between the holding power of the animals in the middle of the bed and at the jetty. All other areas differed significantly from each other.

The mean breaking modulus for byssus threads is 10×10^8 dynes/cm.² It seems logical to assume that the holding power of a mussel is related to the number of threads produced.

In the laboratory, animals were placed in slack (0.002 liters/sec.), medium (0.02 liters/sec.) and fast (0.2 liters/sec.) running sea water. The average numbers of threads produced in 12 hours were 10.6, 15.0 and 16.6, respectively.

Large (4–5 cm. in length) and small (2–3 cm.) mussels were subjected to combinations of 3 salinities and 3 temperatures for 24 hours. The salinities used were 15.9, 31.3 and 46.3‰. Temperatures were 18, 23 and 28° C. Small animals produced more threads than large animals. Large animals produced the greatest number of threads at a salinity of 31.3 under all temperature conditions. At 31.3‰, the number of threads increased as temperature increased.

Small animals produced more threads at 31.3‰ than at the lower and higher salinities. At 31.3 and 15.9 the number increased with increased temperature. At 28° C. the number of threads produced at the high salinity was significantly less than the number at 31.3. At 18° C., the number produced at the low salinity was significantly less than that at 31.3‰.

Electron microscopy of early cleavage stages in Arbacia punctulata. DANIEL A. GOODENOUGH, SUSUMU ITO AND JEAN-PAUL REVEL.

Various stages during the early development of *Arbacia punctulata* eggs were studied in the electron microscope after fixation in cacodylate-buffered Karnovsky's fixative, modified by the addition of an equal volume of sea water and trinitroresorcinol or trinitrocresol to a final concentration of 0.01%. Examination of mitotic figures shows that the chromosomes at metaphase have no particular relationship to the numerous cisternae and tubules of the endoplasmic reticulum found in the mitotic apparatus. During anaphase, however, membranous elements become closely applied to and eventually completely invest the chromosomes. At early telophase, when the chromosomes lose their condensed appearance, they resemble tiny nuclei (chromosome vesicles), each surrounded by an envelope with numerous nuclear pores and containing a nucleolus-like body.

Examination of the periphery of dividing cells at the onset of cytokinesis reveals the presence of numerous parallel filaments, less than 100 Å in diameter, which are intimately associated with the inner face of the plasma membrane in the region of the early cleavage furrow. During the first cleavage these filaments form a belt about 0.1 μ thick and 10–12 μ wide around the equator of the zygote. This belt remains associated with the leading edge and the lateral walls of the furrow as it invaginates, but appears to maintain its original width and thickness during cleavage. In later divisions a narrower filamentous belt is associated with the plasma membrane in the cleavage furrow region.

As cytokinesis proceeds, numerous folds project from the wall of the furrow into the newly formed intercellular cleft. Folds from opposite sides may make contact with each other. At the end of cytokinesis the intercellular space between the newly formed blastomeres becomes largely reduced to a narrow zone between elaborate interdigitations of the cells. In various regions the intercellular space becomes altogether obliterated, suggesting the formation of actual intercellular junctions between the blastomeres.

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Pharmacological studies on electromechanical coupling in a phasic molluscan muscle. MICHAEL J. GREENBERG, ROBERT B. HILL, HIROSHI IRISAWA AND HIROMICHI NOMURA.

The radula protractor of *Busycon canaliculatum* is a twitch muscle; each stimulus to the nerve results in a single phasic contraction. Tetanus tension is regulated both by the frequency of muscle action potentials, and by the level of membrane depolarization. Membrane potentials of the isolated muscles were measured in the sucrose gap apparatus simultaneously with tension. While low potassium solutions hyperpolarized with relaxation, high potassium solutions depolarized with contracture. Resting potentials were 50–55 mV. Acetylcholine (ACh) solutions also depolarized the muscle and produced contracture (threshold, $3 \times 10^{-7} M$). Both electrical and mechanical effects increased with ACh concentration to $10^{-3} M$.

Tryptamine ($10^{-3} M$), alone, produced only about a 4 mV. depolarization together with a just measureable tension increase. However, in the presence of a 75 m*M* KCl contracture, tryptamine induced further tension development but without affecting the depolarization. Tryptamine could not enhance the large contracture resulting from 150 m*M* KCl. Excess calcium, added during a 75 m*M* KCl contracture, did not affect depolarization but induced a small transient tension increase. Caffeine (10^{-3} g./ml.) had a similar though larger effect. Prior soaking in 18 m*M* calcium for 10 minutes greatly augmented an ensuing KCl contracture. Therefore, tryptamine may increase the tension accompanying KCl depolarization by accelerating the availability of calcium as an activator.

Added during a contracture induced by $7 \times 10^{-7} M$ ACh, $10^{-3} M$ tryptamine reduced the depolarization and relaxed tension, although ACh remained in the test solution. Tryptamine had the same qualitative effect at higher ACh levels ($10^{-5} M$), but the relaxation was less rapid. However, the characteristic oscillations of membrane potential and tension were more marked and prolonged during these slower relaxations. This phenomenon suggests a model for the origin of rhythmicity through the oscillation of a membrane made unstable by nicely balanced concentrations of opposing neurohumors.

The morphology of a mechanoreceptor organ of the bivalve, Spisula solidissima. MARY ANN HART.

The receptor ganglion with four sensory regions is suspended between the cardinal teeth of the right valve. In addition, a small anterior sensory region and a small posterior sensory region are associated with the receptor organ. A small wedge-shaped tooth with a fragile laminar process from the left valve interdigitates with the right valve cardinal teeth and stimulates the four sensory regions of the ganglion complex and the small posterior region. A lateral tooth from the right valve stimulates the small anterior sensory region.

Application of mechanical stimuli to the sensory regions of the intact animal results in foot withdrawal. The receptor regions are phasic in their responses to single pulses and to the onset and cessation of continuously maintained pressure from the stimulating probe.

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Determination of rate of polypeptide chain assembly in vivo in Opsanus tau. AUDREY E. V. HASCHEMEYER AND JANICE C. LEVY.

Previous studies have indicated that cold 10°-acclimated toadfish have a greater liver protein synthetic capacity than control (21–22°) fish, when measured at the same temperature. It was also found that aminoacyl transferase activity is elevated in livers of cold acclimated fish. The transferases catalyze steps involved in the binding of aminoacyl-tRNA to ribosomes and its addition to the growing polypeptide chain, and thus can control the rate of chain assembly. In order to determine if this rate differs in cold-acclimated and control fish, an experimental procedure was designed to measure the rate of addition of radioactive amino acids on the ribosomes and the appearance of radioactivity in completed soluble protein. Toadfish were anesthetized and strapped to a fish rack with circulating oxygenated sea water passing over the gills. Five μc. of a mixture of C^{14} amino acids were injected into the hepatic portal vein. After the desired incubation time, from 1 to 8 minutes, the needle was removed, and the liver was excised, homogenized, and fractionated by differential centrifugation. Incorporation of radioactivity into protein was determined in the total homogenate and in various fractions, including the ribosomal pellets and the ribosome-free supernatant. Ratios of these quantities when plotted *vs.* time gave values of t_c, the time of assembly of an average polypeptide chain in liver, independent of the usual individual animal variations. For control fish, t_c was about 6.7 minutes at 22°; with the assumption that an average protein chain in liver is about 50,000 molecular weight, the result compares favorably with data of Dintzis on hemoglobin chain assembly (1.5 minutes per 16,000 MW) in reticulocytes and that of Loftfield and Eigner on ferritin production (6.5 min. per molecule) in rat liver. A faster rate of chain assembly at 22° was found in livers of 10°-acclimated fish.

Calcium activated bioluminescent proteins from ctenophores (Mnemiopsis) and colonial hydroids (Obelia). J. WOODLAND HASTINGS AND JAMES G. MORIN.

The biochemical system responsible for the bioluminescent flashing in *Mnemiopsis leidyi* and three species of *Obelia* (*bicuspidata, geniculata* and *longissima*) has been isolated and partially characterized. In each case the system involves a calcium activated protein similar to that isolated by Shimomura *et al.* from certain hydromedusae. In our experiments the active proteins were isolated by extraction in 0.04 *M* EDTA in 0.2 *M* Tris at pH 10. After dialysis these extracts are stable for at least several weeks. Bioluminescence can be evoked simply by the addition of excess calcium. The light emission occurs as a flash having a duration of about one second. The identity of the energy-yielding chemical step is obscure. The reaction does not utilize or depend upon the presence of molecular oxygen. Only a single component in the extracts is involved. Its molecular weight was estimated to be about 18,300 by gel filtration, this being identical with that of a sample of the active protein from the hydromedusan *Aequorea aequorea.* The material isolated from *Mnemiopsis* is similar in its response to calcium and in its molecular weight, but it has the unusual property of being rapidly inactivated by exposure to light. This feature could account for the fact that the yield of activity in extracts is greatly decreased after exposure of the living organism to light. Such is not the case in *Obelia* even though light may inhibit the *in vivo* bioluminescent flashing.

Microtubules in the axial filament complexes of acoel turbellarian spermatozoa, as revealed by negative staining. CATHERINE HENLEY, DONALD P. COSTELLO AND CHARLES R. AULT.

The mature spermatozoa of both *Childia groenlandica* and *Polychoerus carmelensis* have an anterior nucleus followed by 2 parallel rows of refractile bodies bounded on either side by an undulating membrane. The apparent differences between the two are in their differing lengths and widths: 52 × 4–5 microns for *Childia,* 400 × 1–2 microns for *Polychoerus.* Under certain specific conditions, at least, both spermatozoa are highly motile, progressing by waves passing along each of the two undulating membranes from the tip of the tail towards the head.

As seen in cross-section by electron microscopy, both have two incorporated axial filament complexes, each about 0.18 micron in diameter and with 9 doublet microtubules arranged in a circle; these complexes lack the central pair of the $9+2$ pattern. This offers evidence against an invariable correlation between motility and the presence of the central pair of microtubules. The cilia on the surfaces of both animals *do* have the $9+2$ arrangement.

Late spermatids or spermatozoa of *Polychoerus* were negatively stained with 1% phosphotungstic acid and examined with the Zeiss electron microscope. Large bundles of microtubules were found, containing both doublets and singlets which were bound together at the posterior end by a cap-like structure with definite periodicity. A total of 18 doublets and varying numbers of singlets could be counted. The diameters of the doublets were ±400 Å, those of the singlets ±200 Å, with periodicity in the walls of both of ±75 Å.

In spermatozoa of *Childia* similarly negatively stained and examined by EM, a large number of intact individual axial filament complexes was observed, each with 9 doublet microtubules, all 9 being attached to what is apparently the basal plate of the blepharoplast. The doublets had a repeating substructure in their walls of ca. 150 Å period, and were about 400 Å in diameter. No intact axial filament complexes were observed with more than 9 attached doublets.

Thus far, the posterior cap-like structure binding the microtubules of the axial filament complexes together in *Polychoerus* sperm has not been found in spermatozoa of *Childia,* but further work is continuing, with investigations of negatively-stained microtubules in spermatozoa of other forms, including *Chaetopterus.*

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The effect of electrical stimulation on the action of sulfhydryl reagents in axonal preparations. GILBERT R. HILLMAN AND HENRY G. MAUTNER.

The effects of N-ethylmaleimide (NEM) and parachloromercuribenzoate (PCMB), reagents capable of reacting with sulfhydryl groups, were studied in giant axons of squid, *Loligo pealei,* and in nerve bundles from the walking leg of lobsters, *Homarus americanus.* In order to avoid damaging the nerve membranes, the reagents were applied externally, and the nerve responses were followed using external electrodes.

PCMB and NEM both blocked the nerve response. The action of NEM was irreversible; the effect of PCMB could be reversed completely with 2-mercaptoethanol. NEM was used at 5 and 10 m*M,* PCMB at 0.05 m*M,* and 2-mercaptoethanol at 10 m*M.*

The effects of both reagents were potentiated strongly by electrical stimulation. In the squid axon, treatment with 10 m*M* NEM for ten minutes gave 30% reduction of the size of the action potential; the application of one threshold stimulus per second for thirty seconds resulted in 100% block. Similar effects were seen in lobster nerves at 5 m*M* NEM.

The effects of PCMB also were potentiated by stimulation, both in the lobster and in the squid. Using a small stimulus voltage with the lobster nerve, it was possible to stimulate just a few fibers in the nerve bundle. After 15 minutes of treatment with PCMB all fibers were 50%–70% inhibited. Selective stimulation of these relatively sensitive fibers 6 times a second for 2 minutes gave complete block only of these fibers; the remaining fibers stayed at a constant level of activity until the stimulating voltage was raised above their threshold for another 2 minutes.

These findings are interpreted to indicate that there is a membrane biopolymer containing sulfhydryl groups which are made available for chemical attack only during membrane excitation. It is possible that the sulfhydryl reagents act at more than one site, with some sites being insensitive to stimulation effects.

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Interstitial marine gastrotrichs from Woods Hole, Massachusetts. Part II. WILLIAM D. HUMMON.

A preliminary study of the gastrotrichs from Woods Hole was reported a year ago in this journal. Since then, a one-year ecological study has been completed on Crane's Beach, located at the proximal end of Penzance Point, facing Buzzards Bay. Now included in the list of known gastrotrichs from this beach are all seven species reported last year, as well as three additional species: *Xenotrichula pygmaea* Remane, 1934, *Diplodasys ankeli* Wilke, 1954, and *Macrodasys caudatus* Remane, 1927. The latter three species are here reported for the first time from North America, each having previously been listed among the fauna of both the Atlantic and Mediterranean Coasts of Europe.

Pseudostomella roscovita, noted last year from Nobska and MBL Beaches, has subsequently been found to inhabit a comparable position on Crane's Beach, where it co-occurs regularly with *Tetranchyroderma papii. Turbanella cornuta* has also been observed on Crane's Beach since last August, but occupying a restricted position at 5–10 cm. depth, low-tide elevation. Of the newly reported species, *Xenotrichula pygmaea* regularly inhabits the 5–40-cm. depths at all tide levels, while both *Diplodasys ankeli* and *Macrodasys caudatus* occur only rarely, at 25–40-cm. depths, mid-tide elevations. Tidal elevations in both reports refer to mean high- and low-tide levels, and occurrences refer only to summer observations.

In addition to the ten species indicated above, another five, new or as yet unidentified, have been observed in samples taken from Crane's Beach. A total of 15 species thus have been found in a beach measuring only 15 m. in width from high- to low-tide levels. Data on seasonal patterns and changes in density, dispersion and co-occurrence have formed a major part of the Crane's Beach study. They will be correlated with patterns and changes in physical niche parameters in a future publication.

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Migration of germ cells through successive generations of pallial buds in Botryllus schlosseri. C. S. Izzard.

Berrill (1941) states that in *Botryllus* germ cells originate in the atrial epithelium of the vesicle-stage bud and segregate from the epithelium into the adjacent mesenchyme space underlying the epidermis. In previous light and electron microscopical studies I could find no cytologically differentiated cells in the epithelium nor evidence of cellular segregation from the epithelium. The study has been extended on living sexually-mature colonies and sectioned material.

Germ cells first appear in the vesicle bud, but they are relatively large oocytes (20–40 μ diameter) in a previtellogenic phase of growth. Oocytes in meiotic prophase do not appear until 2 days later following the formation of atrial folds, and gut and pericardial rudiments.

The associated parental bud contains many previtellogenic growth-phase oocytes which, in living buds, prior to the appearance of oocytes in the vesicle bud, are washed from the gonad area into the blood. For approximately 12 hours, large numbers of these oocytes circulate throughout the adults, buds, test vessels and ampullae.

Shortly before the release of oocytes into the blood, a circulatory loop from the parental blood-circulation develops through the vesicle bud. Over a period of 24 hours, previtellogenic growth-phase oocytes passing through the loop settle on either side of the vesicle in the mesenchyme space immediately above the loop. These oocytes mature in this bud generation. The precocious appearance of germ cells and the absence of early oocyte stages in the vesicle bud are thus explained.

From their distribution in sectioned material, one can infer that a population of small (8 μ diameter), mitotic (hence not oocytic) cells, cytologically similar to early spermatogonia, also migrate from the parental gonad area to the vesicle bud. Their appearance prior to that of the testes and meiotic prophase oocytes supports the notion that they represent a primordial germ cell stock.

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Aspects of carbohydrate metabolism in Crustacea. Michael A. Johnston and Frank M. Fisher, Jr.

Hormonal regulation of carbohydrate metabolism by the decapod eyestalk has been investigated in two crustaceans, the fresh-water crayfish *Cambarus* sp. and the marine spider crab

Libinia emarginata. Basal concentrations of hemolymph and hepatopancreatic sugars were determined for normal animals and for those subjected to eyestalk ablation. Reduction in basal levels is noted for several sugars. In the hemolymph the decline from intact to ablated animals is 36% for glucose, 52% for reducing compounds, and 68% for total carbohydrates. Hepatopancreatic levels show similar declines for ablatants: for glucose, 44%; for reducing compounds, 24%; and for polysaccharides, 64.5%. The data suggest an influence on basal sugar concentration by an elaboration from the eyestalk.

Water balance is also affected. Normal *Libinia* show blood volumes of 0.379 ± 0.04 ml. of fluid per gram body weight; the protein content is 0.53 g./ml. of blood. Ablated animals show correspondingly larger blood volumes (0.53 ± 0.33 ml./g.) and a smaller protein content (0.33 g./ml.). Dilution resulting from ablation is clear, but such does not completely account for decreases in sugar levels.

Remarkable differences are noted in a comparison of hemolymph and hepatopancreas polysaccharide content. In the hemolymph, freely-circulating polysaccharides constitute a high 78.5% of total sugar concentrations. In the hepatopancreas, the polysaccharides are a small 6.1% of the total. Acid hydrolysis of hemolymph polysaccharides recovers 60% as glucose, suggesting that the blood rather than the hepatopancreas may be the important sugar storage site in this species.

Cambarus and *Libinia* were injected with exogenous (100–1000 μg.) glucose. Blood samples were removed hourly for five hours and assayed for glucose. Expected glucose-tolerance curves are noted with no significant differences apparent between intact and ablated individuals. The identity of curves indicates that eyestalk hormonal elaborations have no remarkable influence over dispersion of sugar within the blood nor over transport of carbohydrates out of the circulation.

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The biological role of Bugula-type avicularia. KARL W. KAUFMANN, JR.

Very little work has been done to determine the biological role of the avicularia of ectoprocts. The most widely accepted theory is that they are used for defense. However, no one has presented sufficient evidence to demonstrate what the avicularia might specifically defend against. *Bugula simplex* and *Bugula* sp. were intensively studied from a morphological and ecological viewpoint to determine (a) what the avicularia of the two species are best suited to do, and (b) what biological role they actually perform. Structural studies of the musculature, size, strength, and orientation on the colony showed that the avicularia were best suited for grabbing animals with many small appendages and which were in the size range of 1 to 3 mm. Ecological studies of the two species showed that the gammarid amphipods *Corophium insidiosum* and *Jassa falcata* did extensive harm to the colonies by building tubes in them, were of the proper size and shape to be easily caught by the avicularia, and were hindered in their activities by the avicularia. Thus, the primary biological role of the avicularia of *Bugula simplex* and *Bugula* sp. was found to be that of defense against these two amphipods. The widespread distribution of both *Bugula*-type avicularia and tube-building organisms similar to these amphipods makes it likely that this is not a unique association.

The work was carried out under NSF grant GB-7325 to T. J. M. Schopf.

Crustacean pigmentary-effector isohormones. L. H. KLEINHOLZ.

Chromatography of acetic acid extracts of lyophilized eyestalks on G-25 Sephadex columns resolves two zones of pigmentary hormone activity; region 1B contains distal retinal pigment (DRPH) and brachyuran melanophore (MDH) activators, while Peak 4 contains the erythrophore hormone. When Sephadex Peak 1B material is eluted from DEAE cellulose columns with collidine-acetate buffers, followed by 0.1 M HCl, 6 peaks are revealed after alkaline hydrolysis and ninhydrin reaction. The first two of these peaks, conveniently called "alpha-DRPH" and "beta-DRPH," are active. Assay injections of alpha-DRPH from 8 different preparations, in doses ranging from 0.28 μg. to 1.5 μg., result in average DRP indices between 0.150 and 0.233. Tests with beta-DRPH from the same preparations in 0.45 μg. to 2.3 μg. doses give DRP indices between 0.081 and 0.190. An index of about 0.200 characterizes the light-adapted retina, while that for the dark-adapted retina is about 0.050. Alpha-DRPH is resolvable into two peaks (alpha-1-DRPH and alpha-2-DRPH) of activity by gradient elution with acetate buffer from CM cellulose columns. DRP indices of 0.142 and 0.174 result from doses of 3 μg. (alpha-1-DRPH) and 0.39 μg. (alpha-2-DRPH), respectively. Beta-DRPH residues, similarly chromatographed, are separable into several peaks, only the first of which is active. MDH, which disperses the pigment of brachyuran melanophores, consistently accompanies DRPH activity, and appears in the same peaks. Electrophoresis and chromatography of alpha-1-DRPH and alpha-2-DRPH on thin layer silica gel show only one spot reacting to chlorine-tolidine reagent; the melanophore and retinal pigment activities may be due to the same substance.

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Neurophysiology of oculomotor neurons in teleosts. M. E. Kriebel, S. G. Waxman, G. D. Pappas and M. V. L. Bennett.

Oculomotor neurons in several teleosts were studied by intracellular recording. Motoneurons were identified by antidromic stimulation of nerves to extraocular muscles. Electrotonic spread could often be measured between pairs of neighboring cells. Short latency (<0.3 msec.) graded depolarizations were evoked in many cells by graded antidromic stimulation subthreshold for the penetrated cell. These depolarizations presumably resulted from electrotonic spread of antidromic spikes in nearby cells. Depolarizations were not evoked by stimulating heteronymous muscle nerves even where nuclei were close together. Thus, motoneurons innervating different muscles are not coupled. Interneuronal fibers were identified by orthodromic firing without PSP's and absence of spikes in response to antidromic stimulation. These fibers often showed short latency graded depolarizations upon antidromic stimulation of oculomotor nerves indicating electrotronic coupling to motoneurons. Electron microscopy demonstrated "tight junctions" at some synapses on motoneurons. Motoneurons are probably electrotonically coupled by way of presynaptic fibers which synapse on a number of cells. Most motoneuron spikes evoked by eighth nerve stimulation arose sharply from a nearly level baseline and there was little subthreshold PSP, suggesting that the spikes arose from highly synchronous and rapidly rising PSP's or propagated in from dendrites. Spikes evoked by ophthalmic nerve stimulation arose from large slowly rising PSP's, although the apparent firing level was not always constant. Brief, subthreshold spike-like potentials were observed in many cells. Some were due to electrotonic spread of spikes from neighboring cells, since they were associated with an impulse in the efferent nerve and were blocked by antidromic stimulation. Others were not blocked by antidromic stimulation and were probably due to spikes in dendrites or to PSP's. Electrotonic coupling of motoneurons is probably important in mediating the synchronous efferent volleys that can be evoked by orthodromic stimulation. Different modes of impulse initiation may operate in fast and slow eye movements.

Electron microscopy of the excretory vesicle in the cercaria of the trematode, Cryptocotyle lingua. Paul L. Krupa, Gilles H. Cousineau and Arya K. Bal.

In the cercaria of the parasite, *Cryptocotyle lingua,* the excretory vesicle or bladder is a large, saccate organ, located at the caudal end of the body near the junction of the tail. In thin-sectioned specimens fixed in glutaraldehyde and osmium the epithelium of the vesicle appears as a multinucleated syncytium containing numerous secretory granules or droplets, which are reminiscent of zymogen granules. The membrane-bounded granules are circular or ellipsoid in profile and contain a dense amorphous material. Some of the granules, in groups of 4 or 5, tend to be aligned perpendicular to the luminal surface of the vesicle. The number of granules increases as the parasites develop; few granules are found in intraredial stages of cercariae, but in free-swimming cercariae the epithelium of the vesicle is charged with vast clusters of secretory granules, with their enveloping membranes in close apposition to each other. In early embryological stages of cercariae the rough endoplasmic reticulum contains irregularly-shaped saccules, but in free-swimming cercariae the cisternae of the endoplasmic reticulum are arranged in numerous tightly-packed arrays. Other structures in the cytoplasm of the vesicle are Golgi, mitochondria, and myelin-like whorls. Cytoplasmic flaps or rugae project into the lumen of the excretory vesicle and that of the collecting and accessory ducts. Membranous elements are found occasionally in the lumen of the vesicle, but secretion of the granules into the lumen does not seem to occur in intramolluscan or free-swimming cercariae.

The trematode protonephridial system, usually consisting of flame cells, ducts, a vesicle or bladder, and a pore opening externally, has long been assumed to be excretory and osmoregulatory in function on the basis of little or no direct evidence. The presumed role of the excretory vesicle has been the temporary storage of excretory wastes carried to it by ciliated cells and ducts of the excretory tree. On morphological grounds presented in this study, however, the secretory nature of the excretory vesicle epithelium may have some importance in the successful completion of this parasite's life cycle.

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Functionally excitable area of shrimp medullated giant fiber. Kiyoshi Kusano and Matthew M. LaVail, III.

External potential field during impulse conduction along the functionally isolated medullated giant fiber of shrimp (*Penaeus setiferus*), showing a conduction velocity greater than 90 m./sec. at 24° C. without nodes of Ranvier, was recorded with a 1 M NaCl glass capillary electrode. Recordings from most parts of the myelin-covered giant fiber surface showed no action potentials or only small monophasic positive potentials. However, at the points of giant fiber-motor fiber synapses and where giant fiber axons branch into abdominal ganglia, negativity of up to 1 mV. was recorded. By surrounding a single area of synapses or ganglia with a Vaseline partition, a positive-negative or positive-negative-positive potential could be recorded. When physiological saline in the partition was replaced with Na-free, isotonic KCl, or 10^{-7} g./ml. tetrodotoxin-containing saline, the negativity was abolished, and impulse conduction was blocked. Application of these solutions to a myelin-covered area did not block impulse propagation. Fine structure studies of the giant fiber at the area of synapses revealed that the myelin sheath thins and is absent in some small areas. The axon sends a branch to this region. The terminal knob of this branch shows characteristic infoldings and interdigitations with a similarly infolded motor fiber axon. Similar morphology is seen at the sites of contact between the giant fiber axon and the ganglion. Thus functionally excitable areas are present which appear to act similarly to nodes of Ranvier. They make possible impulse conduction at high velocity in shrimp medullated giant fiber. The myelin sheath is very resistive to depressants of excitation and also has a high electrical resistance. Impulse propagation therefore appears to be saltatory.

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Maltase activity in developing embryos and adult tissues of the surf clam, Spisula solidissima. Leonard Laster and Philipp Strittmatter.

As part of a general examination of the association of enzyme activities with the appearance of organ systems during embryonic development of the surf clam, *Spisula solidissima,* in past years we assayed various oxidative and hydrolytic enzymes in embryos and adult tissues. Many of these activities did not increase significantly or changed only slightly. Because the surf clam utilizes carbohydrates as nutriment, in the present study we examined properties of the maltase activity in tissues of the adult clam and determined the appearance of this enzyme activity during the first three days of embryogenesis.

Maltase activity of adult clams is concentrated in the digestive tissues, particularly in the digestive gland. Thus, maltase specific activity in homogenates of digestive gland is approximately 100-fold greater than in homogenates of foot muscle. Unfertilized eggs and early embryos (during the first 24 hours after fertilization) are comparatively low in maltase activity. During the second day of embryonic development there is a rapid, 10-fold, increase in maltase specific activity. This increase coincides temporally with the reported appearance of the esophagus, stomach, intestine, and digestive gland during embryogenesis in this animal. Maltase activity may, therefore, serve as one biochemical marker for gastrointestinal organogenesis. In addition, our observation that digestive gland maltase activity is predominantly associated with intracellular particles suggests that its increase during development of embryos may reflect functional differentiation of a particular organelle in cells of the digestive gland.

We are now isolating the enzyme activity for further characterization of its properties with a view toward utilizing it to study regulatory mechanisms underlying its rapid rise during the second day of development.

Composition and formation of insoluble protein in the dogfish (Mustelus canis) lens. Sidney Lerman, Joan Tuttle and Richard Koser.

The concentration of insoluble protein (albuminoid) in the ocular lens serves as an excellent aging parameter in all species, including the dogfish. Initially, the lens is mainly composed of the three soluble protein fractions (alpha, beta and gamma crystallin) and water (60–70%), but there is a progressive increase in the insoluble protein fraction with aging. The amino acid compostion of dogfish lens albuminoid is very similar to that of the gamma crystallin fraction. The tyrosine content of these two fractions is particularly significant in that it is almost twice that of alpha and beta crystallin, resulting in E_1^1 values of 22–25 for gamma crysallin and albuminoid in contrast with significantly lower values in the other two crystallins. The SH and SS level of gamma crystallin derived from the mature dogfish lens is identical with the corresponding levels in albuminoid, and electrophoresis of solubilized albuminoid (by means of sulfonation) and gamma crystallin on thin layer Pevikon plates show similar R_f values. Precipitin reactions utilizing antibodies to all four lens protein fractions and the individual proteins provide further evidence of a close relationship between the gamma crystallin fraction and the insoluble protein of the dogfish lens. The foregoing experiments indicate that as the lens ages, the albuminoid (insoluble protein) fraction derives mainly from gamma crystallin by means of conformational changes, disulfide bridges between the gamma monomers, and certain other changes in gamma crystallin which enhance the process of polymerization and insolubilization. The latter is probably related to the presence of a fluorescent compound which becomes apparent as the lens ages and is localized to gamma crystallin and albuminoid. Fluorescence and UV spectra and chromatographic analyses indicate that this compound is a derivative of tyrosine and may be a form of bityrosine in which the phenolic groups are substituted rather than free. The fact that this fluorescent compound is present in a specific peptide derived from 6-hour tryptic digests of gamma crystallin would tend to rule out the possibility that it is a breakdown product derived from tyrosine or tryptophan.

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The number of molecules involved in inhibiting either an enzyme or cell division. Robert B. Loftfield and Elizabeth Ann Eigner.

We (*J. Biol. Chem.*, 1968) have shown that the inhibition of an enzyme can be represented by the equation; $\log(1/\mathrm{v} - 1/v) = \mathbf{C} + n \log \mathrm{I}$, where v is the uninhibited rate of reaction; v, the inhibited rate; **C**, a collection of constants; I, the concentration of inhibitor; and n is a small integral number representing the number of inhibitor molecules required to deactivate the enzyme. The value of n is easily determined as the slope of a straight line resulting from a series of experiments in which all factors are constant except for change in I. We showed that the value of n was exactly 1 or exactly 2 for a variety of enzymic reactions, evidence that precisely one or precisely two molecules of inhibitor are required to inactivate alcohol dehydrogenase or amino acid activating enzymes.

We wondered whether much more complex biological reactions can be examined by the same technique. When the accumulated work of Clowes (*Ann. N. Y. Acad. Sci.*, 1951) on the phenol inhibition of cell division of *Arbacia punctulata* is analyzed in this fashion, were find n has the exact value of 1.0 for *p*-aminophenol and dinitro-carvacrol and exactly 2.0 for 2,4,6-tribromophenol, 2,4,6-triiodophenol and 2,4-dibromophenol. There is correspondingly a strong suggestion that there is a vital center involved in cell division that is inactivated by exactly 1.0 or 2.0 molecules of the above phenols. On the other hand, some metabolic poisons such as iodoacetic acid react with a number of amino acids of widely varying function in enzyme systems of widely varying importance. Such poisons would not be expected to yield linear graphs because there is no single target. When the iodoacetate inhibition of *Arbacia* division (Krahl and Clowes, *J. Gen. Phys.*, 1941) is plotted as above, the slope changes from about 1.0 to 4.0 in the range 0.2 m*M* to 33 m*M*.

Supported by USPHS Grant CA 08000.

Stabilization of axopodia by D_2O; a pressure-temperature study. DOUGLAS MARSLAND AND MICHAEL HIRSHFIELD.

Previously it has been shown by Tilney, Hiromoto and Marsland that the axopodia of *Actinosphaerium nucleofilum* become unstable, with concomitant beading and retraction, upon exposure to high pressure (4000–8000 psi). Moreover, the stability of the axopodium as a whole appears to be related to the stability of the complex system of microtubules found in the axial core.

The present work involves the use of two agencies, namely, deuteration and rising temperature. These are known to have a stabilizing influence on the structural integrity of the mitotic apparatus, presumably because of their effects upon the microtubular components.

With increasing concentrations of D_2O, a pronounced increase in resistance to axopodial beading and retraction has been observed. Thus at 20° C., the minimum pressure required to induce complete retraction rose from 5000 psi with no D_2O; to 6000 psi for 50% D_2O; 8000 psi for 60%; 10,000 psi for 70%; and 12,000 psi for 80%.

Experiments still in progress indicate that a further increase in axopodial stability also occurs when the experimental temperature is raised from 20° C. to 25° C. Thus the pressure requirement at the higher temperature has proved to be about 6000 psi in the H_2O medium; 8000 psi for 50% D_2O; and 9000 psi for 60%.

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Differential effects of LAS detergent on reproductive performance of male and female brine shrimp Artemia. D. F. MOFFETT AND D. S. GROSCH.

Linear alkylate sulfonate, the straight-chain biodegradable detergent which is the principal active ingredient in household washing products, was found to reduce reproductive performance of mating pairs of adult *Artemia* at concentration levels reported in polluted waters (A. G. Spencer, 1967, M. S. Thesis, N. C. State Univ.). The present experiment is analogous to the reciprocal cross method of revealing extra-chromosomal inheritance. Young adults were exposed in brine solution containing 5 parts per million of LAS for 8 hours. The time for 50% lethality was 22 hours. Treated and untreated shrimp were paired to give three groups: females treated, males treated, and neither sex treated. Reproductive performance was recorded for 30 days from the appearance of the first brood. Damage *via* the female appeared as significant depression in number of broods, number of zygotes per brood, total number of zygotes, and survival of larvae to maturity. The sex ratio of survivors was normal. Although fewer encysted zygotes were produced, their hatchability surpassed even the control values, suggested pre-deposit selection. The number of females with blocked ovisacs in which resorption of cyst-like material can be observed is consistent with this hypothesis. While the female was productive, the frequency of brood deposit was unchanged. However 6 of the 10 treated females averaged only 3.7 broods while the other 4 females averaged 9.3 broods. Preliminary studies with a continuous recording respirometer indicate that LAS can inhibit oxidative metabolism at such concentrations. This suggests that the agent causes somatic effects as well as cytosomal changes, perhaps dauer modification, in the female gonads.

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Excitatory physiology and localization of bioluminescence in Obelia. JAMES G. MORIN, GEORGE T. REYNOLDS AND J. WOODLAND HASTINGS.

A partial survey of the Woods Hole hydroids has revealed that both the hydroid and medusoid generations of three species of *Obelia* (*bicuspidata, geniculata* and *longissima*) are bioluminescent.

A study of the localization of light emission within the *Obelia geniculata* and *O. longissima* hydroid stage was achieved by photographs taken through an image intensifier. Luminescence was initiated by stimulating across the colony perisarc with fine silver-silver chloride electrodes. These studies show that the stolons, stems and pedicels are luminescent, while the hydranths and gonangia are not. The light emanates from discrete, irregularly spaced spots. Successive pictures show that the spots are in fixed positions along the coenosarc.

Recordings of luminescence from specific areas were made using micromanipulator-held fiber optics with a tip diameter of 0.5 mm. coupled to a photomultiplier recording system. The colonies were stimulated as before. Suction electrodes with tip diameters of 10–50 μ were used to record electrical activity from the hydranths during some experiments. In all three species suprathreshold stimulation at a rate of one or less per second produces a burst of flashes lasting from a few hundred milliseconds to several seconds. These bursts do not appear until after the second or third stimulus. The flashing frequency within this burst ranges between 4 and 16 per second and is highest at the beginning. Successive bursts show marked fatigue in flash number and intensity. The initial flash has a latency of 10 to 20 msec. and all flashes show rise times of 20 msec. and total durations of about 100 msec. Flashes within the burst facilitate markedly and then slowly fatigue. Conduction velocities computed from records of two fiber optics observed from different points within the colony give values between 10 and 20 cm./sec.

Simultaneous records of the electrical activity and luminescence show an accompanying non-facilitating potential of 200–500 μV. and 75 msec. duration with each task. The constancy of spots in successive image intensifier pictures and the phenomenon of luminescent bursts from a small region of coenosarc give indirect evidence for repetitive flashing of individual spots.

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A continuous rate assay for transpeptidating enzymes. R. V. Myhrman and L. Lorand.

Transpeptidases catalyze the nucleophilic attack by amines on activated carbonyl groups such as the γ-amide of glutamine residues. Enzymes of this type are already known to be important in the crosslinking of fibrin structures with γ-glutamyl-ε-lysine bonds in the coagulation of vertebrate blood. Liver transglutaminase (of unassigned biological function so far) and a clotting enzyme ("tissue coagulin"), found in lobster tail muscle, catalyze similar reactions.

To facilitate the search for transpeptidases, in general, we have developed a continuous fluorescence rate assay based on the incorporation of N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide into α-casein. With the transpeptidase catalyzed incorporation of this fluorescent lysine analog into the protein, there is a marked enhancement of the fluorescence of the naphthalene residue ($\lambda_A \backsim 360$ mμ; $\lambda_F \backsim 540$ mμ), as if this chromophore were placed in a more hydrophobic environment. Since α-casein probably contains several incorporation sites, the fluorescence change may represent an overall average. It will be interesting to examine proteins of known tertiary structure and with a single glutamine residue, in place of α-casein, as acceptors.

In the case of the lobster muscle enzyme, a $K_{M,\,app}$ of about 20 *μM* was obtained on the basis of the initial rate of enhancement of fluorescence at different concentrations of the amine; (24° C; pH 7.5; 0.04 *M* Tris; 1.5 m*M* calcium chloride; 0.53 mg./ml. α-casein, Mann).

Aided by grant HE-02212 from the National Institutes of Health and by a grant from the American Heart Association.

Receptor and generator potentials of ampullae of Lorenzini in the skate, Raja. S. Obara and M. V. L. Bennett.

Recording and stimulating electrodes were placed in ampullae while monitoring neural output. In some ampullae negative-going all-or-none spikes were evoked by cathodal stimuli. These receptor spikes usually had thresholds of *ca.* 5 mV., but could appear spontaneously. They reached 20–30 mV. in amplitude and lasted *ca.* 50 msec. In other ampullae cathodal stimuli evoked graded oscillations without a sharp threshold, and stronger stimuli might also evoke spikes. Both types of responses were larger in ampullae than in their canals and probably were generated by receptor cells. Cathodal stimuli increased nerve discharge frequency but impulses had no fixed phase relations to receptor oscillations. Small brief positive-negative potentials could be recorded superimposed on receptor oscillations and were due to nerve impulses, because antidromic stimulation evoked similar potentials. Receptor spikes caused a burst of impulses in an innervating fiber followed by a silent period. Often receptor spikes produced a burst followed by greatly decreased activity in the whole afferent nerve indicating that receptor spikes generated by one ampulla could affect other ampullae. Generator potentials recorded intra-axonally close to ampullae had minimum latencies of 6–9 msec. and could outlast ampullary potential changes. Generator potentials due to brief stimuli recovered following superimposed antidromic spikes which were reduced in amplitude over a time course following the amplitude of the underlying generator potential. These results indicate prolonged transmitter action producing increased conductance. A tentative model accounting for the data is that conventional depolarizing activity of lumenal faces of receptor cells is evoked by cathodal stimuli and this activity depolarizes inner faces, causing transmitter release. The transmitter then depolarizes innervating fibers. Receptor oscillations represent asynchronous and perhaps graded activity of varying numbers of receptor cells arranged in parallel, whereas receptor spikes are due to synchronous and regenerative activity of the receptor cells.

Dogfish erythrocytes: activation of nucleic acid synthesis in a repressed cell. THORU PEDERSON AND SEYMOUR GELFANT.

The presence of a nucleus in the mature erythrocytes of non-mammalian vertebrates raises questions about the nucleic acid metabolism of such cells. We have studied DNA and RNA synthesis in the mature, nucleated erythrocytes of the smooth dogfish, *Mustelus canis,* using cytological and biochemical methods. Dogfish erythrocytes are completely inactive with respect to nucleic acid and protein synthesis, and electron microscopic studies reveal no cytoplasmic organelle systems whatsoever in these cells. However, these fully repressed cells can be activated under certain physiological and experimental conditions.

Cytophotometric measurements of Feulgen-stained nuclei revealed that all mature erythrocytes contain the 2C amount of DNA; nuclei with increased DNA contents were not observed. This lack of DNA synthesis was confirmed by radioautography of cells labeled with DNA precursors. Further, no RNA or protein synthesis was detected in mature erythrocytes by radioautography. These findings indicate that, under normal conditions, the dogfish mature erythrocyte is a completely repressed cell.

Dilution of whole blood samples with either autologous plasma or elasmobranch Ringer's resulted in an activation of DNA and RNA synthesis in mature erythrocytes, as measured both by total isotope incorporation and by radioautography. Dilution experiments were also performed *in vivo,* in which large quantities of blood were withdrawn and replaced immediately with equal volumes of sterile Ringer's. In these experiments an activation of erythrocyte nucleic acid synthesis was also observed.

Following the removal of about 25% of the total blood volume from dogfish, there occurred a rapid activation of DNA and RNA synthesis in circulating mature erythrocytes. This activated state persisted for at least 24 hours after blood removal.

These studies show that the repressed nucleus of the dogfish mature erythrocyte can be activated, and that such an activation may be an important physiological adjustment during blood regeneration.

Supported by research grant GB-2803 from the National Science Foundation.

Effects of an ecdysone on growth and cuticle formation of Drosophila imaginal discs cultured in vivo. JOHN H. POSTLETHWAIT AND H. A. SCHNEIDERMAN.

When the imaginal discs of *Drosophila melanogaster* are implanted into larvae, they metamorphose when the larvae metamorphose. When they are implanted into adult abdomens, the discs may grow, but they do not metamorphose. The present experiments were designed to see whether injection of an ecdysone into an adult fly will cause implanted imaginal discs to metamorphose.

Whole leg discs from mature third instar larvae were injected into fertilized adult females. The hosts then received single or repeated injections of ecdysterone (=20-hydroxyecdysone) in 10% ethanol in Ringer. In a typical experiment, one group of flies bearing implants received either 7.2 or 720 μg. of ecdysterone/g. fly weight in a single dose, or in six equal installments over a period of eleven days. In none of the singly injected flies did the discs grow significantly or metamorphose. In contrast, multiply treated implants increased in size more than three fold, and most secreted some cuticle, but failed to metamorphose.

To cause metamorphosis, repeated doses of higher concentrations of ecdysterone were necessary. Thus 3600 μg./g. given over an eleven-day period in three injections caused metamorphosis in thirteen of thirteen implants. These metamorphosed implants were completely covered with cuticle, and formed bristles, claws, tibial sensory organs, sex combs, and sensilla trichodea.

These results indicate the following: 1) lack of ecdysone in uninjected adult flies accounts for the absence of metamorphosis in implanted discs. 2) Ecdysterone is inactivated rapidly in the adult. 3) To cause either growth or metamorphosis, ecdysone is needed as a sustained stimulant, not merely as a trigger (hence the effectiveness of repeated doses). 4) Low concentrations of ecdysone stimulate the enlargement of discs, whereas, 5) high concentrations stimulate cuticle secretion and metamorphosis.

This discovery of a simple chemical method of regulating the growth and metamorphosis of imaginal discs promises to simplify developmental studies with *Drosophila.*

A comparative analysis of the control of the white chromatophores in the fiddler crab, Uca pugilator, from Woods Hole, Massachusetts, and Panacea, Florida. K. RANGO RAO AND MILTON FINGERMAN.

Saline extracts of central nervous tissues of *Uca pugilator* from the Woods Hole area were known to evoke white pigment concentration, but it was not known whether they also contained a white pigment-dispersing substance. Subsequent investigations of *Uca pugilator* from Panacea revealed that saline extracts of optic ganglia, sinus glands, supraesophageal ganglia, and thoracic ganglia evoked white pigment dispersion alone. However, acetone fractionation revealed the presence of white pigment-dispersing and -concentrating substances in each of these tissues. To learn whether the corresponding tissues from Woods Hole crabs also have both substances, the tissues were extracted in acetone. In each instance the acetone-soluble fraction contained the white pigment-concentrating substance whereas the acetone-insoluble fraction contained the dispersing substance. However, the circumesophageal connectives of the crabs from Panacea and Woods Hole contain the white pigment-concentrating substance alone. Additional studies were performed with Panacea crabs. Saline extracts and the ethanol-soluble fraction of the eyestalks evoked nearly identical white pigment-dispersing activity, but had no white pigment-concentrating activity. The question arose then whether the white pigment-concentrating substance is absent in these extracts or masked by the antagonistic action of the white pigment-dispersing substance. Gel filtration of the ethanol extracts on a column of Sephadex LH-20 in ethanol revealed the presence of both substances. Exposure of the ethanol-soluble fraction of the eyestalks to heat or isopropyl ether resulted in destruction of the white pigment-dispersing substance. Although the white pigment-dispersing substance in lyophilized aqueous extracts of the eyestalk was thermostable, it was almost completely destroyed by exposure to isopropyl ether. Such treated extracts evoked white pigment concentration, thereby revealing that the white pigment-concentrating substance was present in the aqueous and alcoholic extracts but was masked by its antagonist, the white pigment-dispersing substance.

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Cell recognition in an echinoderm, Asterias vulgaris. CAROL L. REINISCH AND FREDERIK B. BANG.

Echinoderms, particularly asteroids, are ideal animals for study of cell interaction since they afford easy access to the coelomic cavity for continuous sampling, and clear microscopic observation of the papillae (and hence circulating cells) under the dissecting microscope.

Asterias vulgaris has an average circulating amoebocyte count of 10^6 cells/ml. of coelomic fluid. Amoebocytes of *Arbacia punctulata* were used as a challenge inoculum. Because of their pigmentation, interaction between *Arbacia* and *Asterias* cells was readily observed. After the initial *Arbacia* inoculum, periodic samples for counting were withdrawn from the *Asterias* in warmed N-ethyl maleimide (NEM) which prevents invertebrate cells from sticking to one another.

Initial clearance of *Arbacia* amoebocytes as unbound or non-amoebocyte-associated cells occurred within 120 minutes in 13/16 experiments. Characteristic clumping, in response to the challenge *Arbacia* cells, was seen in the papillae. There was a sharp initial drop in the number of circulating free host amoebocytes which occurred within 1 minute after injection. The clumping, when correlated with the disappearance of free *Arbacia* cells, was interpreted as rapid cell recognition of foreign cellular material.

To see whether NEM might alter cell recognition *in vivo,* two sets of experiments were done. In one set, sea stars were pretreated with 2×10^{-4} *M* NEM before inoculation with *Arbacia* cells; in the second, *Arbacia* cells were treated with NEM before inoculation. Neither pretreating the host animal nor challenge cells altered normal host cell recognition.

An aspect of NEM's effect was alteration of amoebocyte behavior on glass. Normally these cells sent out pseudopodia within seconds after contact with a glass surface. Both viable amoebocytes from an NEM-treated *Asterias* and amoebocytes which had phagocytosed an *Arbacia* cell remained rounded up to 1½ hours. Normal cells or cells which had phagocytosed a carborundum particle, in comparison, spread normally.

Electron microscopical studies on "myosin" from molluscan smooth muscle. ROBERT V. RICE, G. F. ELLIOTT AND A. STRACHER.

Standard methods requiring several days for preparing purified myosin from striated muscle failed to give recognizable particles when applied to a variety of molluscan smooth muscles. The preparations dissolved in volatile buffers of ammonium acetate were sprayed or streaked onto mica, shadowed with PT and coated with carbon in a vacuum evaporator. These preparations when examined in the electron microscope showed only 50 Å globules in contrast to similar preparations from striated muscle which routinely show 1600 Å rods with bulbous heads 200 × 50 Å. Isolation of protein in the presence of the antibiotic tetracycline did not change the results. In contrast to the standard methods a new procedure, which uses sucrose-gradient centrifugation, was used. This procedure, originated by Dr. Peter Hardwicke of the Biophysics Department of King's College, London, WC2 (personal communication, to be published shortly), gives purified fractions from muscle in a matter of hours. Fractions from various levels of the sucrose-gradient centrifugation were dialyzed against 0.6 *M* ammonium acetate and prepared for electron microscopy as described above. Fractions were also prepared by negative staining. The 50 Å globules were found in a band about 1 cm. from the tube top, and were relatively free of other fractions including F-actin filaments and paramyosin ribbons. The pellet contained thin paramyosin ribbons (a sub-structure of the paramyosin filaments) and some F-actin. When the pellet was dissolved in 0.1 *M* ammonium acetate the paramyosin ribbons were studded with 50 Å globules which were generally absent if the pellet was dissolved in 0.6 *M* ammonium acetate. From these experiments we tentatively conclude that thick paramyosin filaments, which from x-ray diffraction are known to contain closely packed rods (1400 Å long) of tropomyosin A, also have as part of their structure, associated small (50 Å) globules. The globules are very labile and are easily detached in 0.6 *M* salt solutions. At the surface of the filament the small globules probably interact with F-actin and represent the "cross bridges" between thick and thin filaments in molluscan smooth muscle.

Activity level and cardioregulation of the northern sea robin, Prionotus carolinus (L.). JOHN L. ROBERTS.

Innervation of teleost hearts by parasympathetic efferent fibers has been suggested recently to be a pathway for cardioregulation only at low levels of activity. This is because the release of vagus "braking" action by bilateral vagotomy and atropine block at the heart does not result in cardioacceleration at higher activity levels or with steady swimming at moderate velocities. A corollary of this view is that parasympathetic activity is nearly always turned off and therefore of no significance to active species that characteristically are continuous or nearly continuous swimmers. Results that follow bear out these contentions with regard to the bottom-dwelling, inactive sea robin, *P. carolinus.*

Like most bottom fish, standard level heart rates of sea robins (about 70 cpm) as measured from ECG recordings are considerably higher than ventilation rates (5–40 cpm) obtained at 21.5° C. (visual, EMG and branchial cavity pressure recordings). Also, ventilatory pauses and rate and amplitude changes, and an associated transitory bradycardia are pronounced as a result of visual disturbances.

Thermal stress applied by warming (1° C./min.) restrained, lightly anesthetized fish (MS 222, 1/23,300) from acclimation temperatures between 20° and 22° C. until the brain (deep body temp.) reaches 27° to 28° C. results in a hypoxic bradycardia concurrent in time and degree with progressive respiratory failure. Both the visually induced and the hypoxic bradycardia are largely relieved by pericardial injection of 0.2 to 0.3 ml. atropine (10^{-3} M). Atropine injection also elevates standard level heart rates (from 69 to 107 cpm), but seems to have no effect upon cardiac rates of fish subjected to enforced moderate swimming (1 body length/sec.; 9- to 11-inch fish). In fact, heart rate accommodation seems to saturate (about 120 cpm) at a low swimming velocity (undetermined) above which no further rate change occurs.

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Do ploidy and cytoplasmic volume influence the cleavage rate of sea urchin eggs? RONALD C. RUSTAD.

Two different approaches have been used to evaluate the consequences of reducing the amount of nuclear or cytoplasmic material on the cleavage rates of the eggs of *Arbacia punctulata* and *Lytechinus pictus.* In a series of studies, conducted in collaboration with S. Yuyama and L. Rustad, eggs were cut into nucleate and anucleate halves or quarters. After fertilization the diploid half and quarter-cells divided at the same time as the whole cells, and the haploid cells divided significantly later. However, there was no comparable increase in the duration of the second, third, or fourth mitotic cycles of the haploid cells.

The delay found in the first division cycle of the haploid cells might be a special consequence of the fact that the male pronucleus is initially condensed and not in equilibrium with the cytoplasm. Therefore, the "Hertwig effect" was used to prevent the male pronucleus from participating in the normal mitotic cycle of whole eggs. The sperm were exposed to massive doses of γ-rays (200 to 800 Kr.). At the lower doses almost all of the fertilized eggs underwent multipolar mitoses after several hours cleavage delay; however, a small percentage divided into two cells at the same time as the diploid eggs. High doses often immobilized the sperm. In successful experiments with the higher doses from 50% to more than 90% of the eggs which were fertilized with the irradiated sperm completed each of their first four cleavages at the same time as the controls. These experiments demonstrate that a biologically haploid cell containing a female pronucleus, instead of a male pronucleus, divides at the normal rate. Therefore, neither cytoplasmic volume nor ploidy *per se* determines the rate of cleavage of sea urchin eggs.

These studies were supported by the U. S. Atomic Energy Commission and the Office of Naval Research.

Mercenene clam extract: effects on the population kinetics of an experimental carcinoma and a non-neoplastic tissue. SISTER ARLINE CATHERINE SCHMEER, O.P.

The cell population kinetics for the 4-day-old transplantable Krebs 2 solid carcinoma (a non-metastatic tumor) and the cryptal progenitor compartment of duodenum from tumor-bearing CF1 mice has been determined by utilizing pulse label radioautographic techniques reported at these General Scientific Meetings last year. This report describes the effect of mercenene clam extract on the population kinetics of the above named tissues. The Krebs propagation and transplantation was identical to that reported previously. The tumor and duodenum (a non-neoplastic tissue) were prepared for radioautographic analysis by utilizing standard procedures. A series of experiments consisted of 7 daily injections of mercenene into mice with 4-day-old Krebs carcinoma. Experimental and control animals were sacrificed 24 hours after each daily injection of clam extract, so that the last groups killed had received 7 doses of mercenene, and had tumors 11-days-old. ^{3}H-TdR was administered 30 minutes before sacrifice. The DNA synthetic index (DSI) (% labeled tumor cells/each 1000 non-labeled tumor cells scored), and mitotic index (MI), were then calculated. Preliminary results indicate that (1) mercenene is inhibiting the tumor cells during the G_1 phase of the cell cycle by preventing the cells from entering S (DNA synthetic period), (2) those cells in G_1 when the clam extract was administered were destined to die approximately 3 days later, and (3) those tumor cells not in G_1 were unaffected until, or unless, they were in G_1 when mercenene was injected, (4) the percentage labeled nuclei declined for a period equal to the time it takes the cells to die, and (5) the percentage of labeled cells increased as the non-DNA synthesizing population was eliminated. There was no significant difference between the MI of the experimental and control groups of Krebs tumor. The DSI and MI for the duodenal cryptal population demonstrated no significant variation between the experimental and control animals for any given time interval. The conclusion, then, is that mercenene, administered daily for 7 days, has no effect on the kinetics of the progenitor compartment of duodenum of tumor-bearing mice. The clam extract does act as an oncolytic agent against the cells of the Krebs solid carcinoma by preventing the cells from entering S. As reported previously, in *in vitro* studies on HeLa and human amnion (non-neoplastic) cell lines, the extract is non-toxic to amnion cells. It is extremely significant to have an anticancer drug that is selective in killing only neoplastic cells both *in vitro* and *in vivo*.

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The control of molting in arthropods by steroid hormones. HOWARD A. SCHNEIDERMAN AND A. KRISHNAKUMARAN.

Insects are the only arthropods in which the control of molting is well-understood. A group of steroids—the ecdysones, thought to be produced by the prothoracic glands—cause molting in almost all insects upon which they have been tested. Ecdysones have also been isolated from a crustacean and have proven active in insects. However, no reports exist of successful efforts to induce molting in arthropods other than insetcs with ecdysones. This report demonstrates that ecdysones also control molting in four diverse classes of arthropods besides insects.

The ecdysone that was employed in most experiments was ecdysterone (=20-hydroxy-ecdysone), a steroid which has been isolated both from Crustacea and insects. As experimental animals we selected representatives of the major arthropod subphyla and classes: *Limulus polyphemus,* the horseshoe crab, Class Merostomata; *Araneus cornutus,* a spider, Class Arachnida; *Armadillidium vulgare,* a terrestrial isopod and *Procambarus* sp., a fresh-water crayfish, Class Crustacea. Animals were maintained under conditions in which spontaneous molting was minimal. They were usually divided into 3 groups: experimental animals which received an injection of hormone in an appropriate Ringer of 10% ethanol; injected controls which received only the solvent; and uninjected controls. The effects were unambiguous: injection of 3 to 50 μg. of ecdysterone per g. live weight stimulated molting in all species of arthropods examined. In many animals molting occurred almost synchronously. The response was proportional to the dose: the larger the dose, the sooner the molt. Similar results could be obtained by injecting other ecdysones (inokosterone, alpha ecdysone, ponasterone A) and by topical application of a methanolic solution of ecdysterone.

From these data it appear likely that ecdysones were employed by the common ancestor of the major arthropod groups in the late pre-Cambrian, at least 200 million years before vertebrates evolved. Probably this particular chemical control of molting evolved simultaneously with the development of a chitinous exo-skeleton and may exist in some non-arthropod groups.

Skeletal wall structure of a calcified bryozoan (Phylum Ectoprocta). THOMAS J. M. SCHOPF AND DOROTHY F. TRAVIS.

Knowledge of how the mineralized skeleton of bryozoans forms is essential for the evaluation of phylogenetic schemes derived from examination of calareous wall layers. Discussion of this topic at the First International Conference on Bryozoa in 1968 showed that it is one of the most important current problems in the study of bryozoa. We have therefore given attention to the manner of calcification in *Schizoporella unicornis*, a representative of the most common group of bryozoans (Order Cheilostomata, Suborder Ascophora Vera).

A model of the calcification of this species can be reconstructed from the following cross-sections.

(1) Sections through the basal, lateral, proximal and distal walls into the main body cavity: cuticle, calcitic layer(s), epidermis, and peritoneum.

(2) A section through the frontal wall and compensation sac into the main body cavity: cuticle, epidermis, peritoneum, coelom, preitoneum, epidermis, aragonitic layer, calcitic layer, cuticle, compensation sac, cuticle, epidermis, peritoneum and coelom of the main body cavity.

The coelomic space above the compensation sac is continuous with that of the main body cavity through the proximal and lateral pores (areoles) of the frontal surface. The prominent pores through the calcitic and aragonitic layers are filled by epidermis and are attached to the cuticle layer that is immediately above the compensation sac. We envisage the function of these tissue plugs to be that of structural supports which help to suspend the compensation sac during its formation by invagination from the frontal wall and during deposition of the calcitic and aragonitic layers of the frontal wall.

A somewhat similar relationship of the calcified skeleton to soft tissues, including the extension of the coelom into the frontal layer above the compensation sac, was illustrated by Calvet (1900: pl. 7) for a species referred to *Microporella*.

The influence of actinomycin-D on distal-hydranth regeneration in Tubularia crocea. IRWIN SINGER AND JOHN D. PALMER.

The regeneration of new hydranths on isolated hydrocaulus segments has been divided into arbitrary, well-defined morphological stages for the sake of descriptive convenience. The purpose of the study reported here was to determine the role of RNA synthesis in the completion of each of these stages. Actinomycin-D, an inhibitor of DNA-dependent RNA synthesis, was used for this purpose.

Seven- to eight-mm. hydrocaulus segments, removed 3 mm. below the hydranth, were placed in Millipore-filtered sea water containing 0.5, 1.5, 2.5, 5.0, 7.5, or 10.0 μg./ml. actinomycin-D, for 4 hours at 14° C. Between 15 and 30 animals were subjected to each concentration. After this treatment they were washed, placed in fresh sea water, and distal-hydranth regeneration observed at 24-hour intervals for 6–7 days. In control animals, distal-hydranth regeneration was completed by 72 hours. Actinomycin-D had no effect on coenosarc healing or endodermal-ridge regression, but after these stages there was inhibition of further differentiation: inhibition increasing with increasing concentrations. In concentrations up through 2.5 μg./ml., regeneration was retarded, but by 96 hours at least 88% of the individuals had completed regeneration (*i.e.*, reached the emergent stage). At 5.0 to 10.0 μg./ml. never more than 36% of the animals reached the emergence stage, even by 168 hours.

Because of the very pronounced effects starting at 5 μg./ml., the following experiments were undertaken with this concentration. Two to four hours after transection, healing of both ends of the hydrocaulus segments was complete and at this time they were treated with actinomycin for 4 hours; no inhibition resulted. If, however, after healing, the *proximal* end of the hydrocaulus was opened again, then a 4-hour treatment with actinomycin produced an inhibition curve identical with that obtained in the first experiments described above. The inhibition curve remained unchanged until actinomycin was administered through the open proximal end at 22 hours after transection; a 4-hour treatment at this time produced a mitigated inhibition curve.

This work was supported by National Science Foundation grant GB-5045 to J.D.P.

The effects of temperature and various biological substances on mucociliary function of intact Nudibranchiata. Prantika Som and F. B. Bang.

Evidence from our current studies on the mucociliary system of the footpad of intact Nudibranchiata, a shell-less opisthobranchiate mollusc, suggests that the increased viscosity of mucus plays a role in decreasing mucociliary transport rate at higher temperatures. With rise of temperature from 0 to 11° C. there was a concomitant rise in mucociliary transport rate reaching a maximum of 0.78 mm./sec. at 11° C., beyond which a gradual decrease in rate was accompanied by a greater viscosity of the mucus produced. Good ciliary vibration was still apparent up to 31° C. if the overlying sticky mucus was first removed. That the increased viscosity of mucus due to higher temperature decreased the rate of transport seemed to be confirmed by use of a mucolytic agent, N-acetyl cysteine, which increased the rate about 25% at temperatures above 11° C. Below 11° C. little variation from the untreated animal was seen.

A crude preparation of cholera toxin, known to affect mucus secretion in vertebrates, was employed in our system. Exposure of animals for 30 minutes at 21° C. in a concentration of 1 mg./ml. of this material accelerated the transport rate from pre-treatment average of 0.25 mm./sec. to 0.38 mm./sec. over the next 30 to 40 minutes. This was accompanied by increased secretion of mucus. There was a sudden fall to nil rate continuing for 8 to 12 hours followed by an equally sudden increase in rate to levels in excess of those seen before exposure to the toxin and persisting for 5 to 6 days. During this latter period the mucus secreted was very thin. Most animals, treated or normal, did not survive longer, but 2 animals, exposed to toxin, survived 10 days after treatment and showed a return to near normal transport rate, though the mucus continued to be thin.

The use of pilocarpine, a well-known stimulant of mucus secretion, increased the transport rate in normal animals at various temperatures but failed to stimulate toxin-treated animals. Thus, cholera toxin apparently causes a persistent lesion of mucus cells demonstrated by: (a) increased and persistent flow of thin mucus; (b) failure to respond to pilocarpine; and (c) an aberrant increase in rate with increasing temperatures.

Supported by a grant from the Tobacco Research Council.

Nicotine-induced alteration of DNA and cell membranes in gametes of Arbacia punctulata and its bearing on human lung cancer. Carl Caskey Speidel and Ralph Holt Cheney.

In an extensive series of experiments, gametes of sea urchins were subjected for from 1 to 24 hours to nicotine solutions of 9 different strengths ranging from 0.5 to 0.00002 molar. Nicotinized gametes were then fertilized with normal gametes of the opposite type. Development was studied through the stages of cleavage, blastula, gastrula and pluteus.

Strong treatments of spermatozoa that still allowed fertilization could cause visible abnormalities and death of the young zygotes in any of the stages of cleavage before motility appeared. *Moderate* treatments allowed development to gastrulation or pluteus formation. Abnormalities were apparent also in these zygotes. Skeletal and ciliary deficiencies with associated deviations in swimming were particularly conspicuous. *Weak* treatments caused no perceptible abnormalities.

Eggs were subjected to similar nicotine treatments and fertilized with normal spermatozoa. Ensuing abnormalities were similar to those indicated above, although less strong treatments were sufficient to induce abnormalities of like degree. Motion picture records were made.

Since abnormalities of similar nature were induced in the two sets of experiments, we believe that the same underlying mechanism has been altered in nicotinized spermatozoa and eggs. The nicotinized spermatozoan head which enters the egg is composed almost entirely of haploid chromosome material. This combines with the normal haploid chromosome material of the egg, thus bringing about an abnormal diploid condition which then is responsible for the resultant abnormalities exhibited by the progeny. Fundamentally, this indicates to us that DNA, the chief constituent of chromosomes, has been altered by nicotine.

Furthermore, a nicotinized egg may suffer alteration of its cell membrane in such a manner as to allow polyspermy. Our observations suggest that nicotine may alter the cell membrane, cilia and DNA of respiratory tract cells in man. Such alterations would certainly favor the development of lung cancer in heavy cigarette smokers.

Supported by Grant RH 00325–02 to C. C. S. from the Environmental Health Division of the USPHS.

Pigment migration within fiddler crab melanophores. JAMES R. STEWART AND JONATHAN P. GREEN.

Hormonally and osmotically induced melanin migration in *Uca pugnax* was studied in a variety of inhibitor or activator solutions. Two bio-assay systems were used: perfusion of isolated legs with the test solution and incubation of isolated abdomens in the test solution. Advantages of the isolated abdomen assay over the isolated leg assay are its convenience and the assurance of uniform distribution of the test solution to all melanophores. Comparable results were obtainable with either assay system. Melanophores were staged according to the scheme of Hogben and Slome.

Ouabain ($1.4 \times 10^{-4} M$), an inhibitor of sodium-dependent active transport, in combination with melanophore-dispersing hormone (MDH) in sea water, had no inhibitory effect on melanin dispersion in either legs or abdomens. Ouabain in the absence of MDH had no dispersal effect on concentrated melanin. Similar results were obtained with 2,4-dinitrophenol ($5.0 \times 10^{-4} M$), an oxidative phosphorylation uncoupling agent.

Mersalyl ($0.1 M$), a sulfhydryl bond inhibitor, in sea water reduced the rate of MDH-mediated melanophore expansion by 39% over a three-hour period. Mersalyl in 100% and in 80% sea water did not effect pigment dispersion.

Dinitrophenol ($5.3 \times 10^{-4} M$) in 200% sea water caused a 20% decrease in the rate of melanin contraction over a one-hour period. Similar results were obtained with ouabain ($1.4 \times 10^{-4} M$) in 200% sea water.

Calcium chloride and magnesium chloride ($0.6 M$) increased the rate of melanin contraction over that of 200% sea water by 30% and 28%, respectively, over a one-hour period.

These results suggest that sulfhydryl bond formation may be involved in the action of melanin-dispersing hormone. It is unlikely that oxidative phosphorylation or a sodium-dependent active pump process alone is responsible for melanin dispersion. On the other hand, the available evidence points towards active ion extrusion followed by water efflux as a mechanism for melanin concentration.

Supported by National Science Foundation Grant #GY-4304 to Brown University for undergraduate research, by NSF Grant GB-7261 (to J. P. G.) and by NSF Grant GB-3447 to the Department of Invertebrate Zoology, Marine Biological Laboratory.

ATPase and actin combining properties of "myosin" from molluscan smooth muscle. A. STRACHER, ROBERT V. RICE AND G. F. ELLIOTT.

Attempts to prepare myosin from the opaque portion of the adductor muscle of *Mercenaria mercenaria* by the standard method used for vertebrate striated muscle have failed to produce a protein with the known enzymatic properties of myosin. For this reason, the method of Hardwicke, utilizing a sucrose gradient, was used in order to localize and characterize the fraction containing the ATPase activity. After completion of the density gradient run, the bottom of the tube was punctured with a needle and ten 2-ml. fractions were collected. Analysis of each tube for enzymatic activity showed that the third fraction from the top of the tube contained the ATPase activity. This fraction was dialyzed against 0.1 M ammonium acetate, pH 7.0, in preparation for shadow casting. Only 50 Å globules were seen in the electron microscope from two separate experiments. Assuming an $E^{1\%}_{280} = 7.0$, the specific activity was equal to approximately 0.5 μmoles Pi/mg./min., comparable to myosin from vertebrate striated muscle. The activity was quite readily lost if the protein was allowed to remain at 4° C. for longer than 6 hours. Dithiothreitol did not protect against this loss in activity. The dependence of ATPase on both pH and Ca^{2+} was similar to that of myosin from skeletal muscle. If the homogenization and sucrose gradient were carried out in the presence of 0.6 *M* KCl, the pellets consisting of paramyosin ribbons obtained at the bottom of the tube were devoid of ATPase. The pellet from the 0.1 *M* KCl run, however, had a significant amount of ATPase. These results are consistent with the findings described in the abstract by Rice *et al.* Preliminary experiments suggest that an interaction between rabbit skeletal F-actin and molluscan smooth muscle "myosin" occurs, as evidenced by a disappearance of the ATPase from tube 3 when F-actin is added to the 0.1 *M* KCl homogenate before layering on the sucrose gradient.

Studies on the life-history of Distomum pyriforme Linton, 1900. Horace W. Stunkard.

Stunkard (1967) reported on the life-cycle and developmental stages of a digenetic trematode whose metacercarial stages occur in medusae. Ophthalmotrichocercous cercariae develop in rediae in *Anachis avara.* They penetrated and persisted as unencysted metacercariae in *Gonionemus vertens* and *Chrysora quinquecirrha.* When fed to fishes, sexually mature worms were recovered from the scup, *Stenotomus chrysops.* Adult worms are very similar morphologically to *Distomum pyriforme* Linton, 1900, described from the pyloric ceca and intestine of the rudderfish, *Palinurichthys perciformis,* and tentatively they are assigned to that species. The studies have been continued; the number and location of the penetration-glands and flame-cells of the cercaria have been determined. Eight pairs of penetration-glands are situated in the lateral areas between the ocelli and the acetabulum. On each side, ducts from three cells pass forward, lateral to the ocellus, and those of five cells pass mediad of the ocellus. All open at the anterior end of the body. Details of the excretory system have been worked out. The pore is terminal, preceded by a sphincter which closes the uroproct. Anterior to the uroproct there is a pulsatile bladder which is continued anteriorly by a tubular reserve vesicle, filled with spherical concretions, that extends to the level of the pharynx. From the anterolateral faces of the bladder, collecting ducts, lined with cilia, pass forward, lateral to the digestive ceca. Anterior to the acetabulum each divides into anterior and posterior branches. Each branch bears three sets of flame-cells, four in each set and the formula is $[(4+4+4)+(4+4+4)]$. Eggs from the worms have been embryonated; the miracidia develop and emerge in 8 to 10 days at laboratory temperature; they have ocelli with conspicuous lenses, long cilia, and swim rapidly. Life-history data clarify the systematic position of *D. pyriforme.*

Investigation supported by NSF GB 3606, continuation of G 23561.

Localization of bioluminescence in the marine dinoflagellate Pyrocystis lunula Schütt using an image intensifier. Elijah Swift and George T. Reynolds.

Cultures of *P. lunula* were entrained on a 12:12 LD cycle. The non-motile bioluminescent lunate cysts were examined during scotophase in small chambers with water immersion lenses. During scotophase the cyst's plastids migrate to distal areas and facilitate observation of the centrally located nucleus and the perinuclear area, which is *ca.* 25 nm. in diameter and contains obvious starch grains. Macroflashes were stimulated by electrical impulses delivered to electrodes placed near the specimen on the microscope stage. Miniature flashes and glows were associated with chemical and mechanical damage to the cysts. The microscope output was focused on the photosensitive cathode of the image intensifier at magnifications from 20 × to 200 ×. For viewing macroflashes, the image intensifier gain was 9.0×10^5. For viewing the steady glow, the gain was *ca.* 1×10^5. By means of dark field illumination, the structural features of the specimen were photographed through the same microscope-intensifier system, so that the structural sources of the luminescence could be identified.

The photographs obtained indicate that the electrically stimulated flash (macroflash) is produced in the entire perinuclear area. Miniature flashes were localized in several different parts of the perinuclear area. These miniature flashes occurred in a protoplast after the introduction of proprionic acid to its medium. The steady glow was observed to come from the entire perinuclear area. On the photographs, the nucleus appears as a dark area. The steady glow was presumed to be caused by damage to the cyst since the glow, $>2 \times 10^4$ quanta sec.$^{-1}$, is much brighter than spontaneous glow of *P. lunula* cysts. Thus the photographic records provide evidence that the macroflash, miniature flash, and glow are all produced in the small area of the protoplast of *P. lunula* near the nucleus.

Supported in part by AEC contract AT(30-1)-3406 and in part by NSF Grant No. GZ 259.

Fertilization-induced increase of trypsin-like activity in sea urchin "membrane" during embryogenesis. WALTER TROLL, ALBERT GROSSMAN AND SARAH CHASIS.

The mechanism by which protein synthesis and cellular differentiation are initiated by the entry of the sperm into the egg has remained a central problem of biology. Monroy and his co-workers have shown that the ability of ribosomes from unfertilized eggs to engage in protein synthesis is enhanced by treatment with trypsin. This report is concerned with the induction of a trypsin-like enzyme as part of the fertilization process. The enzyme is measured using the synthetic substrates tosyl-arginine methyl ester (TAME) and acetyl lysine methyl ester (ALME). Crystalline trypsin hydrolyzes these substrates, preferring TAME over ALME by eight to one. An enzyme attacking these substrates was isolated from *Arbacia punctulata* eggs by freezing and thawing five times then washing with Millipore-filtered sea water, sonicating and centrifuging. The residue represents the membrane fraction and contains the enzyme. Eggs were fertilized and samples taken at hourly intervals. A 10-fold increase of activity was noted over a 20-hour period. The enzyme differed from trypsin in that it attacked ALME and TAME equally well. Pre-incubation with Actinomycin D (20 μg. per ml.) completely abolished the increase. Tosyl lysine chloromethyl ketone (TLCK), a specific inhibitor of trypsin, inhibited the membrane enzyme. The presence of 0.002 M TLCK prior to and during incubation inhibited the division of *Arbacia;* at 0.0002 M, development stopped at blastula. This suggests two roles for the enzyme: (1) the activation of protein synthesis, and (2) the activation of genome.

Work supported by GM-13728, Core Grant BSS-ES 00014 NCI-CA 06989 and Allied Chemical Corporation. A. G. is a Senior Postdoctoral Fellow of the New York Heart Association.

The responses of granule cells in the cerebellum of Mustelus canis. NAKAAKIRA TSUKAHARA, HELENA TÁBOŘÍKOVÁ AND JOHN C. ECCLES.

The selachian cerebellum differs from the cerebella of higher vertebrates in that the granule cells are concentrated in two large medial cords. Mossy fibers pass to these cords *via* the stratum fibrosum. The axons of granule cells, the parallel fibers, pass up to the molecular layer and then transversely, so making synapses with the dendrites of Purkyně and stellate cells. Purkyně cells are absent over the medial part of the granule cell cords, so a microelectrode there records selectively field potentials produced by granule cells. A mossy fiber volley in the stratum fibrosum evokes in the granule cell cords an initial diphasic potential followed by a slow negative wave, which are attributable, respectively, to mossy fiber impulses and the excitatory synaptic potential induced thereby in granule cells. With increase in the mossy fiber volley these potentials become larger and granule cell spikes are superimposed upon the slow wave. Evidently spatial summation is required for evoking this discharge. A second stimulus through the same electrode reveals that mossy fibers recover fully from refractoriness in about 5 msec. and produce a large second EPSP. This temporal summation of EPSPs causes impulse discharge when single volleys are ineffective, and such facilitation persists for over 70 msec. When parallel fibers are stimulated directly, impulse propagation occurs along the transverse course of the parallel fibers with a conduction velocity of about 0.2 m./sec. These impulses propagate to the granule cells antidromically, the evoked spike potentials being observed in the granule cell cord. With double stimulation it is found that the recovery of antidromic invasion of granule cells occurs earlier than with Purkyně cells, beginning at 5 msec. and with full recovery by 20 msec. No inhibitory action of presumed Golgi cells has been found, otherwise the investigations summarized in these two papers indicate that physiologically the neurones parallel those of mammalian cerebellum.

This work was supported by the Grass Foundation.

Oxygen-linked dissociation of hemocyanin II. A proposed model. K. E. VAN HOLDE AND HENRY A. DEPHILLIPS, JR.

As has been shown in a previous abstract, the aggregation state of squid hemocyanin is a function of the per cent oxygenation. Any satisfactory model must explain both the dissociation upon partial oxygenation and the reassociation upon complete oxygenation.

Since each 19S subunit contains about sixteen oxygen binding sites (from known stoichiometry), there should exist a distribution of levels of oxygenation among the set of subunits, at any given value of average oxygenation. If noncooperative binding is assumed, as a first approximation, this can be shown to be a binomial distribution. We now further assume that subunits carrying more than a critical number (n_1) but less than a second critical number (n_2) of bound oxygens are incapable of entering into 59S aggregates. It is then possible to calculate the per cent dissociation to be expected at any overall level of oxygenation. With the choice $n_1 = 10$, $n_2 = 15$, excellent agreement is obtained between theory and experiment.

This model further predicts that at low overall oxygenation the 19S component will be most highly oxygenated, whereas at high oxygenation the 59S component will be slightly more oxygenated. These predictions have been checked by use of the scanner attachment to the analytical centrifuge. Measurement of the optical density of the two boundaries at 345 mμ yields the concentration of oxygenated protein; on the other hand, the total protein concentration in each boundary can be measured from integrated schlieren patterns. The results of experiments at both low and high levels of oxygenation are in accord with the predictions of the model.

This research was supported by Public Health Service Grants Nos. HE-11671 and HE-12326.

Ribosomal RNA cistrons in single and multinucleolate oocytes. W. S. VINCENT, H. O. HALVORSON, H. R. CHEN AND D. SHIN.

The multinucleolate oocyte of amphibians has been shown to contain reiterated ribosomal RNA (rRNA) cistrons to the extent that the germinal vesicle contains more DNA ascribable to these cistrons than to the entire remaining genome. The question arises as to whether this amplification of ribosomal cistrons is a phenomenon only of amphibian oocytes, or whether it exists in other multinucleolate and uninucleolate forms.

We have examined the ribosomal cistron content of DNA isolated from red blood cells and ovarian tissues containing immature oocytes of the striped bass, *Roccus saxatalis,* a form with multinucleolate oocytes; we have also examined DNA from the sperm, eggs and ovarian tissues of the starfish, *Asterias forbesii,* which contains uninucleolate oocytes. Ribosomal cistrons were detected by RNA-DNA hybrid formation on nitro-cellulose filters with appropriate controls of temperature, incubation time, rRNA saturation levels, salt concentration, fixation of DNA to filters and competition by homologous rRNA.

All DNA species examined demonstrate a heavy satellite region detected by rRNA hybridization following equilibration centrifugation in cesium chloride. In the starfish DNA a satellite band was detected with the Model E ultracentrifuge at $d = 1.710$, with a main band at $d = 1.689$. Determination of rRNA binding to DNA gave the following results (in μμg. rRNA bound /μg. DNA): striped bass rbc DNA: 0.20, ovarian DNA: 2.34; starfish sperm: 0.37, ovarian DNA: 0.35.

From these data we conclude that although the rRNA cistrons are normally redundant in these two forms, amplification of the cistrons is found only in striped bass which has multinucleolate oocytes, not in the uninucleolate starfish oocyte. This suggests that nucleolar number in oocytes may be a function of ribosomal gene amplification.

Supported by Grants from NIH: GM-11480 and AI-1459, from NSF: GB-3273 and B-1750.

The electroretinogram and spectral sensitivity of the common scallop. GEORGE WALD AND EDWARD B. SELDIN.

The eye of the common or bay scallop, *Aequipecten irradians,* contains two retinas, proximal and distal, each sending a branch to the optic nerve. Hartline showed that the proximal retina discharges impulses in the optic nerve only when light is turned on or up, the distal retina only on turning light down or off.

The electroretinogram (ERG) in response to flashes or continuous light consists potentially of two waves. At low intensities in the dark-adapted eye the first wave appears alone, its latency about 100 msec. and peaking at about 200 msec. At higher intensities and on light adaptation it is accompanied by a second, slow wave, peaking at about 300 msec. In dark-adapted eyes the first wave may be cornea-positive or -negative. In partly light-adapted eyes it is always cornea-negative, as is always the second wave. These on-responses presumably originate in the proximal retina. The ERG displays no off-response, hence apparently no signal from the distal retina.

The spectral sensitivity of dark adapted eyes was measured—the number of photons per 18-msec. flash that at each wavelength evoked a constant amplitude of ERG (20 or 50 μvolts). A simple curve results, with average λ_{max} 490–495 mμ (range 480–507 mμ). This is not changed in shape or position in the spectrum by adaptation to bright red or violet light. It seems therefore to be based upon a single visual pigment, resembling in spectrum the rhodopsins earlier extracted from squid and cuttlefish retinas.

This investigation was supported in part by a National Science Foundation grant to G. W.

Optical changes associated with nerve excitation and membrane polarization. A. Watanabe, I. Tasaki and L. Carnay.

Nerves from lobster, spider crab and squid fluoresce when stained with such dyes as acridine orange or 8-anilinonaphthalene-1-sulfonic acid. The intensity of fluorescence, detected at a right angle to the incident light, was found to increase transiently when the nerve was stimulated electricially. At the peak of excitation, the increase was within the range of $(1–5) \times 10^{-4}$ times the intensity of the background fluorescent light. The time-course of the increase was roughly parallel to that of the action potential. Similarly, the birefringence and turbidity of the nerve were shown to exhibit transient changes during nerve excitation. (The results of the present birefringence and turbidity studies are consistent with those reported recently by Cohen *et al.*). Optical changes of the nerve could also be produced by altering the ratio of the univalent-divalent cation concentration of the medium, by temperature changes and by electrotonic polarization of the nerve. The sign of the optical signals produced by cathodal polarization was the same as that produced by the action potential; anodal polarization produced optical changes of the reversed polarity. Within a certain range of polarization intensity, the optical effect of polarization and that of the action potential were additive. This finding suggests that the optical changes of the nerve are a rather complex phenomenon, involving various anatomical structures in the vicinity of the axonal membrane.

The effect of high hydrostatic pressure on the mechanical properties of the membrane of the sea urchin egg. Lewis Wolpert, Douglas Marsland and Michael Hirshfield.

There is evidence that high hydrostatic pressure can solate and weaken gel-like structures within cells. It is thus of interest to determine its effect on the mechanical properties of the cell membrane since it has been suggested that there is a gel-like cortex beneath the plasma membrane which makes a significant contribution to its mechanical properties. The mechanical property measured was the resistance to deformation when the eggs are compressed. The eggs of *Arbacia* were compressed by placing a small plate made either from a fragment of coverslip (120 μ thick) or Meline plastic sheet (50 μ thick) on a small group of eggs (20 to 100) so that the eggs supported the plate. The deformation of the eggs was determined by measuring their increased diameter. The diameter of the eggs was about 70 μ and a force of about 8×10^{-3} dynes/egg increased this to about 110 μ. Compressed unfertilized eggs from which the jelly coat had been removed by acid sea water showed no change in diameter when subjected to 12,000 lb./in.^{2} for 30 minutes. The mechanical properties of the cell membrane of unfertilized eggs appear to be unaffected by high hydrostatic pressure. When compressed fertilized eggs, from which the fertilization membrane had been removed by urea treatment, were subjected to pressure of about 10,000 lb./in.^{2} there was a significant increase in cell diameter. This increase was such that it appeared as if the resistance to deformation of the membrane were halved. Surprisingly after a few minutes the diameter decreased again indicating a contraction at the membrane even while under high hydrostatic pressure. Release of the hydrostatic pressure resulted in a further reduction in diameter and thus contraction at the surface, the final diameter sometimes being less than that before the hydrostatic pressure was applied.

The effect of light on the dogfish eye. SEYMOUR ZIGMAN AND SUSAN J. BAGLEY.

The influence of strong light over protein synthesis in dogfish (*Mustelus canis*) ocular tissue was observed *in vitro* using elasmobranch Ringer's medium. Corneas, lenses, and retinas of medium-age dogfish were obtained fresh and incubated at 20° C. under 95% O_2:5% CO_2 in Ringer's solutions containing 1 μc./ml. of C^{14} amino acid mixture in the dark or illuminated by a 40W circular cool white fluorescent lamp with 475 and 570 mμ wave-length maxima. The intensity of light in the incubation chamber was 500–600 ft. candles.

Incorporation of C^{14}-amino acids into the insoluble (1200 *g* residue), particulate (105,000 *g* residue), and soluble proteins was measured by counting TCA precipitates of these fractions after removing residual radioactivity by suction filtration. Specific activities were calculated from the results of liquid scintillation counting in Bray's solution of hyamine hydroxide solubilized protein, and the Lowry (phenol) protein analysis.

Amino acid incorporation into the particulate fractions of cornea epithelial cells, lens epithelial cells, lens proper, and whole retina between 7 and 22 hours in the light was reduced to approximately ½ that of the tissues kept in the dark. Soluble protein synthesis was reduced by about 50% also in lens epithelium and retina. Inhibition of amino acid incorporation into soluble proteins was not observed.

When total collagen was extracted from corneal stroma, amino acid incorporation was found to be inhibited by 20% during 24 hours of illumination. No effect was noted upon incorporation into soluble lens proteins. No appreciable conversion of soluble to insoluble proteins took place.

Whole retinal rods isolated by soaking and shaking isolated retinas in ice-cold Ringer's medium (which contains urea at 0.3 *M*), were incubated up to 24 hours in the light and in the dark. By 24 hours, a loss of 65% of protein synthetic activity was observed in illuminated rods, concomitant with appreciable cell damage. Partial recovery of protein synthesis could be achieved by returning the rods to the dark after 6 hours.

The mechanism of light inhibition of protein synthesis in ocular tissues is under investigation.

Supported by ONR/CNA grant (University of Rochester) and Rochester Eye Bank.

Comments on the extraction of Thalassopsammon from intertidal and subtidal sands. DONALD J. ZINN.

The removal of the interstitial fauna from intertidal and subtidal sands continues to be a relatively difficult technique in the efficiency and reliability of extraction of a desired taxon or taxa. Although the more commonly used methods of extraction, conveniently categorized as puddling, mechanical, salinity gradient, bubbling and elutriation, elutriation and bubbling are apparently the most satisfactory means for finding maximum group representation (qualitative), these methods as originally described are only occasionally suitable for quantitative extractions. For example, ciliates, tardigrades, turbellarians and gastrotrichs are removed with greatest efficiency by sea-ice (salinity gradient); mystacocarids, copepods, oligochaetes, polychaetes and kinorhynchs are best extracted with bubbling, while ostracods and nematodes may be optimally removed by elutriation.

Recent and present investigations have indicated strongly that (1) extraction efficiencies of different higher and lower taxa exhibit different rates; (2) the presence of excess detritus, non-capillary sediments bacteria, yeasts and algae can alter extraction rates radically to the point of prevention; (3) soft-bodied and contractile forms are most efficiently extracted with modifications of a standard method best for the class; and (4) the most reliable method for testing efficiency is to place a known number of identified individuals into a measured amount of sterilized natural sand and extract with the developed method of choice. Uhlig, using the sea-ice method he developed, indicated an efficiency of 90% for ciliates and 75% for turbellarians, while Fenchel, also working on ciliates, found a variation of 70% to 90% for the same method depending on the manipulation of the animals.

Special methods must be developed by each investigator for extracting interstitial taxa of special interest. Examples of variants on basic methods include introduction of narcotizing agents (magnesium chloride, propylene phenoxytol, etc.), bubbling with a gas (carbon dioxide, nitrogen, etc.), elutriation with diluted and/or heated sea water and varying the temperature of the sample before extraction.

Contribution No. 164 of the Systematics-Ecology Program, Marine Biological Laboratory, Woods Hole, Massachusetts. Grateful acknowledgment is made for assistance provided by the University of Rhode Island Grant-in-aid No. 132–151.

ERRATA

The following corrections should be made in the paper by Lofts, Pickford and Atz (1968), *Biol. Bull.,* **134**: 74–86:

Page 75, line 17—*Group III.* 10° C.

Page 76, line 20—Group II.

Page 78, Table I—For II, read III; for III, read II.

Page 85, Rasquin and Hafter. *Anat. Rec.* **111.**

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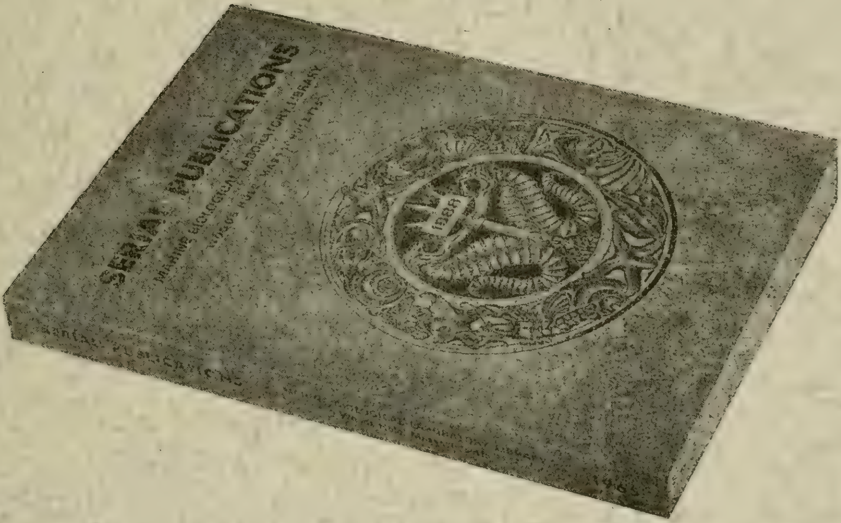
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CONTENTS

Page

CHUANG, SHOU-HWA
The larvae of a discinid (Inarticulata, Brachiopoda) 263

HINSCH, GERTRUDE W.
Reproductive behavior in the spider crab, Libinia emarginata (L.) ... 273

KELLY, MAHLON G.
The occurrence of dinoflagellate luminescence at Woods Hole 279

LAMBERT, GRETCHEN
The general ecology and growth of a solitary ascidian, Corella willmeriana 296

MCPHERSON, B. F.
Feeding and oxygen uptake of the tropical sea urchin Eucidaris tribuloides (Lamarck) 308

OHTAKI, TETSUYA, ROGER D. MILKMAN AND CARROLL M. WILLIAMS
Dynamics of ecdysone secretion and action in the fleshfly Sarcophaga peregrina 322

PETERSON, MARGARET K., AND JOHN BUCK
Light organ fine structure in certain Asiatic fireflies 335

ROPES, JOHN W.
Reproductive cycle of the surf clam, Spisula solidissima, in offshore New Jersey 349

SUMMERS, WILLIAM C.
The growth and size distribution of current year class Loligo pealei. 366

YONGE, C. M.
Form and habit in species of Malleus (including the "hammer oysters") with comparative observations on Isognomon isognomon .. 378

Abstracts of papers presented at the Marine Biological Laboratory 406

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SEXUAL RESPONSES OF ADULT MALE AEDES AEGYPTI USING THE FORCED-COPULATION TECHNIQUE[1, 2]

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Our present knowledge concerning the sexual responses of the male Yellow Fever Mosquito *Aedes* (*Stegomyia*) *aegypti* Linnaeus is based largely on the studies of Roth (1948), Spielman (1964), Jones and Wheeler (1965a, 1965b), Spielman *et al.* (1967), and Craig (1967). According to Roth (1948), adult males in cages copulate repeatedly and remain in a permanent mating state throughout their lives. He observed that one virgin male caged with 16 virgin females for 30 minutes copulated 30 times and made 10 additional attempts to do so. He found that males would copulate with 50-minute-old females. Jones and Wheeler (1965a), using the forced-copulation technique, reported that males would copulate with 4 to 10 virgins within 10 to 20 minutes. Wheeler and Jones (1960) found that while males could be forced to copulate with newly emerged females, they were not inseminated. Lea (1967, 1968) found this to be true for young caged females, and showed that this was influenced by the corpora allata, and was temperature- and strain-dependent. According to Gwadz (1967), females in a cage copulate repeatedly but do not become inseminated until they are 40 to 48 hours old.

VandeHey and Craig (1958) reported that multiple insemination occurred in caged groups of *Aedes aegypti;* Craig (1967) subsequently noted this was true for matings which occurred over a short period of time. Jones and Wheeler (1965b) discovered that males did not force-copulate with inseminated females, and Ludlam (1965) confirmed this. George (1967) reported that while males repeatedly copulate in cages, only the first mating was effective. Craig (1966, 1967) showed that this was due to the male accessory gland component of the semen from the first

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[2] Based on a thesis submitted by the first author to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Master of Sciences, 1968.

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insemination. After implanting male accessory glands into virgin females, Craig obtained a 25% insemination rate using forced-copulation and no insemination with those placed in a cage with fresh males. Spielman *et al.* (1967) reported that when once-mated females were paired with males in a cage 47% of them copulated again, and those which remated very rapidly ejected the semen from the bursae.

It is evident that much remains to be learned about the sexual responses of *A. aegypti* under both forced and cage mating conditions. This paper is concerned with the details of the sexual responses of *A. aegypti* males to very young virgins or to previously copulated females, using the forced-copulation technique, and also includes tests of male potency.

The specific terms which will be used in this paper need to be defined at the outset. *Copulation* refers to the placement of the aedeagus into the upper vagina of the female. *Precoital time* extends from the initial second of the presentation of the male to the beginning of aedeagal emplacement. *Coital time* is the period from the beginning of aedeagal emplacement to its withdrawal from the vagina. *Insemination* is the placement of sperm and male accessory gland materials in the bursa and is followed by spermathecal filling and retention of the male accessory gland material. *Fertilization* is restricted to the placement of spermatozoa in the egg.

MATERIALS AND METHODS

The mosquitoes of the Bangkok strain were reared at 27° C. and 70% relative humidity. The larvae generally pupated on the sixth and seventh days after hatching. Pupae were sexed by examining their external genitalia with a stereomicroscope at 16 ×, using the criteria of Christophers (1960). Virgin adults of each sex and of known ages were also obtained by removal and separation of the sexes as they emerged. The adults were maintained on 5% sucrose. Test lots of adults were kept either in 28 cm. square wire cages or in standard U. S. pint cartons with a fine net cover on top, and were supplied with sucrose on cotton pads. The induced copulation techniques of McDaniel and Horsefall (1957) and Wheeler (1962) were employed; however, none of the insects were decapitated. All forced-copulations (forced-matings) were with mosquitoes that had recovered from the nitrogen anesthesia which was used in the pinning procedure. Females caged with males (sex ratio 1:1) for 2 days were assumed to be already inseminated when used for subsequent forced-copulation tests, and will be designated as *cage-copulated* or *cage-mated* females. All males were offered only once to a female and then were discarded except in the potency tests. Sperm checks of females were made by examination of the spermathecae and/or the bursa at 430 ×.

RESULTS

1. *Male responses to young virgin females*

Forty-eight-hour-old males were presented to 171 young females of nine precisely known age groups (0, 1, 2, 3, 4, 8, 12, and 24 hours ±1%). The young females often interfered with the copulatory attempts of the males by kicking with their legs (chiefly the metathoracic legs), and when this happened the legs were amputated at the midpoint of the femurs.

TABLE I

Coital times of young virgin Aedes aegypti (L.) females which were force-copulated with virgin males 48 hours old. Cases of prolonged coitus are not included.

Age of females	Inseminated females			Uninseminated females		
	Number of copulations	Coital time, seconds		Number of copulations	Coital time, seconds	
		range	mean		range	mean
0	3	21–724	58.3	1	6	6.0
1 hour	3	18–49	34.3	2	4–6	5.0
2 hours	4	33–70	47.8	2	8–10	9.0
3 hours	3	7–120	55.7	0	—	0
4 hours	6	13–200	62.8	2	6–11	8.5
8 hours	11	40–160	85.1	1	120*	120.0
12 hours**	1	40	40.0	1	16	16.0
12 hours	1	115	115.0	1	4	4.0
16 hours	10	10–134	70.4	1	4	4.0
24 hours**	13	30–398	113.8	2	4–12	8.0
24 hours	9	11–99	31.6	1	7	7.0
	64	7–398	71.4±7.7***	14	4–120	7.5± 1.0***

* Male ejaculated prematurely, datum excluded from mean of total coital time.
** Test performed at two different times.
*** Mean based on total data.

The male responses to these females were either (1) rapid copulation, (2) seizure of the female's cerci and then not copulating, (3) no visible reaction at all, or (4) withdrawal from the female terminalium.

The only visible response of the females to the males was withdrawal of their

TABLE II

Copulation and insemination rates of young virgin female Aedes aegypti (L.) which were force-mated with virgin males 48 hours old

Age of females	Number used	Copulations		Inseminated		Uninseminated		Prolonged coitus	
		Number	%	Number	%	Number	%	Number	%
0	16	4	25.0	3	75.0	1	25.0	0	0
1 hour	15	5	33.3	3	60.0	2	40.0	0	0
2 hours	15	6	40.0	4	66.7	2	33.3	0	0
3 hours	15	4	26.7	3	75.0	0	0.0	1	25.0
4 hours	21	10	47.6	6	60.0	2	20.0	2	20.0
8 hours	16	14	87.5	11	78.6	1	7.1	2	14.3
12 hours*	10	2	20.0	1	50.0	1	50.0	0	0
12 hours	15	3	20.0	1	33.3	1	33.3	1	33.3
16 hours	15	12	80.0	10	83.3	1	8.3	1	8.3
24 hours*	18	15	83.3	13	86.7	2	12.5	0	0
24 hours	15	10	66.7	9	90.0	1	10.0	0	0
	171	85	49.7%	64	75.3%	14	16.5%	7	8.2%

* Test performed at two different times.

terminalia. Very young females emitted droplets from their ani and these often interfered with the male's clasping of the cerci and prevented copulation. This was observed primarily with 0- to 1-hour-old females, and was seldom seen in females 8 or more hours old.

The individual coital times of inseminated females ranged from 4 to 398 seconds, with a mean of 71.4 ± 7.7 seconds, compared to a range of 4 to 16 seconds and mean of 7.5 ± 1.0 seconds for the uninseminated females (Table I). From 20% to 87.5% (85/171 average of 49.7%) of the females force-copulated, and 33.3% to 90% (65/85 average of 75.3%) were successfully inseminated (Table II).

Prolonged forced-copulation was observed with 7 out of 85 pairs of mosquitoes. Most of the males were unable to free themselves at will by even the most strenuous efforts. Only one male, after copulating for 360 seconds, succeeded in freeing himself after struggling for 208 seconds. When the female was dissected 45 minutes later, sperm were seen *only* in the bursa. The other six pairs were *in copula* so tightly that manual stretching tore their abdomens apart rather than separating their terminalia.

TABLE III

Precoital and coital times of force-copulated Aedes aegypti (L.) males in 4 age groups; each male was offered to 15 virgin females of the same age

Age of males	Number used	Number of copulations	Precoital time, seconds		Coital time, seconds	
			range	mean	range	mean
1 Day	10	58	1–171	19.7	3–45	18.5
3 Days	10	68	1–103	16.2	3–66	20.9
5 Days	10	72	1–43	10.2	4–55	20.9
7 Days	10	89	1–105	18.3	4–57	20.3
	40	287	1–171		3–66	
Means		7.2 ± 0.5		16.0 ± 1.2*		20.2 ± 0.7*

* Mean based on total data.

2. *Male potency and variability of response to virgin females*

Adults of precisely known ages (1-, 3-, 5-, and 7-day-old lots ±4%) were used to test the responses of 10 individual virgin males (per lot), each individual being presented in sequence and at known intervals to 15 virgin females, or to as many as would be accepted using the forced-copulation technique. Each of the 4 tests required about 6 hours to complete. The responses of the males were subjectively graded as either (1) feeble, (2) moderate, (3) active, or (4) highly active attempts to copulate, or as (5) withdrawal of their terminalia from those of the females. The first 4 responses were qualitatively assessed by the following activities of the males: (1) the extent of tarsal contact with the female, (2) the degree of flexion of the male's terminalium into the female, (3) the speed and number of attempts to copulate, (4) the forcefulness of cercal clasping, and (5) the extension of the paraprocts with eversion of the aedeagus. Withdrawal of the male's terminalium included a pushing of the female with his tarsi and a flexion of the terminalium away from that of the female.

The precoital times of the 40 males ranged from 1 to 171 seconds, with a mean of 16.0 ± 1.2 seconds. The coital times ranged from 3 to 66 seconds, with a mean of 20.2 ± 0.7 seconds (Table III). The males copulated with 287 out of 600 females (47.8%). The number of females acceptable to individual males ranged from 2 to 15, with a mean of 7.2 ± 0.5.

Most of the females were not accepted sequentially by the males; only one out of the 40 males used accepted all 15 females, and this individual did not accept 2 additional females. This highly potent male's initial precoital and coital times were 21 and 24 seconds, respectively. His final precoital and coital times were 3 and 4 seconds, respectively. In these experiments, 6 or more females were accepted sequentially by 19 out of 40 males (47.5%), and 8 or more females were accepted sequentially by 7 out of 40 males (17.5%). Only 2 out of 40 males (5%) copulated sequentially with less than 3 or more than 10 females.

The age of the mosquitoes apparently did not influence either the precoital or coital times (Table III). The number of copulations by 5- and 7-day-old males was significantly higher than that by 1- and 3-day-old males (p less than 0.01). The increase in the number of copulations of 3-day-old males *versus* 1-day-old males and of 7-day-old males *versus* 5-day-old males was significant (p less than 0.01).

TABLE IV

Responses of Aedes aegypti (L.) males in 4 age groups; each male was offered to 15 virgin females of the same age

Male age	Withdrawal responses	Coital responses					Total responses
		Feeble	Moderate	Active	Highly active	Row totals	
1 Day	5	3	29	15	11	58	63
3 Days	13	20	22	15	11	68	81
5 Days	1	30	24	13	5	72	73
7 Days	3	28	28	23	10	89	92
	22 7.1%	81 28.2%	103 35.9%	66 23.0%	37 12.9%	287 92.9%	309

Presentation of a virgin female to a male resulted in definite responses to the female in 309 out of 600 males (52%). In the other 287 (48%), the males showed no recognizable response to the females. Of the responses that did occur, 7.1% (22/309) were withdrawals and 92.9% (287/309) were coital responses. The degree of the coital responses were as follows: feeble, 28.2% (81/287); moderate, 35.9% (103/287); active, 23.0% (66/287); and highly active, 12.9% (37/287) (Table IV).

3. *Forced copulation of virgin versus cage-mated females*

One hundred virgin and 100 cage-mated females were each individually offered a series of one to 5 males, in 5 lots of 20, using the forced-copulation technique. If copulation occurred, no additional males were presented. All the mosquitoes were 48 hours old ±3 hours.

Among the virgins, 95% force-mated for 6 to 76 seconds, with means for the 5 replicates ranging from 22.8 to 29.9 seconds, and with an overall mean of 26.7 ± 1.3 seconds; 81 out of 95 virgins (85.3%) accepted the first male offered, and 10 out of 95 accepted the second male (10.5%).

In great contrast to the above, only one out of the 100 cage-mated females could be force-copulated, and this one did so for only 3 seconds.

Fifty of those virgin females which had previously been successfully force-copulated were again presented to virgin males for a second time, 12 hours after the initial force-copulation. Only 5 out of 50 of them (10%) force-copulated a second time. Their coital times ranged from 26 to 55 seconds with a mean of 28.8 ± 6.8 seconds. However, 48 out of 50 concurrent virgin controls (96%) force-copulated. The coital times of these controls ranged from 12 to 58 seconds, with an overall mean of 25.0 ± 1.3 seconds.

Discussion

Aedes aegypti females which are less than 24 hours old can be inseminated by force-mating (this paper), but none are inseminated at this age within a cage (Lea, 1967, 1968; Gwadz, 1967). Spielman *et al.* (1967) suggested that (1) semen is altered during or immediately after insemination of virgin females and that (2) semen is lost, if not so altered. Thus, it is possible that very young females mating in a cage do not yet have the ability to alter semen, and therefore do not retain it. Since the aedeagus blocks the genital orifice during forced-mating about 5 times longer than during cage-mating, it is possible that the retention of the semen in young force-mated females is due to the fact that it has time to become self-altered, whereas the semen in young cage-mated females flows out of the bursae before it has time to become self-altered. It is known that the male accessory gland material alone gels very rapidly in saline *in vitro* (Jones and Wheeler, 1965a). Since Jones (unpublished data) noted that the bursal walls lack the machinery of secretory cells, the bursa probably does not secrete anything which would alter the semen.

It is also conceivable that the bursae of very young females differ in several respects from those of older females. The very young females are swollen by air and fluids in the gut (Christophers, 1960) and this might distort the bursae. If during the relatively prolonged period of forced-mating, the pairs were more firmly united than during the briefer period of cage-mating, then a longer and firmer union might permit the force-copulating male to fill the bursa of a very young female, whereas a shorter and less firm union might not permit the free male to do so adequately.

Further, there may be a difference in the coital mechanics between forced-copulation and cage-mating of the young females. The descriptions of coitus by Jones and Wheeler (1965b) and Spielman (1964) differ enough to suggest that this is a very real possibility. The male may be unable to grasp the young female properly in cage-mating but can do so when force-copulated. If the cage-copulating male does not grasp the young female correctly, then (1) the male may only appear to cage-copulate with the young female but not ejaculate, or (2) the aedeagus of the free male may not be able to attach to the dorsal vaginal valve properly so that the ejaculate is not deposited in the bursa. The phallotreme's position must be exactly within the coital cavity of the inverted upper vagina or the female will not be inseminated properly (Jones and Wheeler, 1965b).

The occurrence of prolonged coitus in cage populations of *Ae. aegypti* has never been reported, yet it was observed in 7 out of 85 (8.2%) force-copulations with young females. This phenomenon may provide a means of determining if the aedeagus blocks the opening to the vestibule (the funnel through which sperm enter the spermathecal ducts). The factors involved in the termination of coitus are not well known. It is believed that the aedeagus overextends and that the connection with the dorsal vaginal valve is easily broken. The aedeagus is then free and pushes against the male's own anal cone. The female then dislodges the male with her legs (Jones and Wheeler, 1965b). In prolonged forced-coitus, the gelation of the male accessory gland material might hold the aedeagus *in situ.*

Very evident in this study was the high degree of variability of the sexual responses of males that had been reared under nearly identical conditions and which were given presumably similar stimuli. Sometimes a male would not copulate with one virgin female but would with a different female. The coital responses shown by individual males appeared to vary between females. Some individual males are certainly more potent in that they are highly active and will copulate sequentially more than 6 times. Although it could be argued that a series of virgin females differ substantially in their attractiveness to a given male, this seems unlikely. Often, in separate tests, females that were actively refused (as indicated by withdrawal of the male's terminalium) by different males were subsequently acceptable to other males. The mean precoital times of 16.0 ± 1.2 seconds for forced-copulation are short compared to the means of 34.1 ± 4.2, 45.0 ± 4.7, and 47.5 ± 6.5 seconds reported for mating in lantern chimneys (Spielman *et al.,* 1967). In the latter study, the time required for flight and tarsal contact probably accounts for the longer interval. The coital time (mean 20.2 ± 0.7 seconds) of the males repeatedly force-copulated agrees with the 19.7 ± 6.4 seconds mean reported by Spielman (1964) for cage-copulated males that had been previously mated twice.

The mean coital time of 26.7 ± 1.3 seconds for 48-hour-old virgins that were force-copulated is higher than the means of 13.7 ± 3.2 seconds (Spielman, 1964), 17.6 ± 0.5)Spielman *et al.,* 1967), and 13.2 seconds (Jones and Wheeler, 1965a) for 3- to 7-day-old virgins that were cage-mated.

The mean of 25.3 ± 0.8 seconds of Ludlam (1965) and the mean of 26.7 ± 1.3 seconds in this study for coital times by forced-copulation agree; they differ from the longer mean of 31.3 ± 1.6 seconds reported by Jones and Wheeler (1965a).

This study strikingly supports the hypothesis that in the Bangkok strain of *Ae. aegypti,* cage-mated females cannot be subsequently force-copulated with males. It was not possible to force-copulate 99 out of 100 cage-mated females. This poses the question of whether the male can distinguish an inseminated from a virgin female. Clasping reflexes were generally present but aedeagal contact was never made with the cage-mated females when the forced-copulation technique was used. Ludlam (1965) did not observe even the clasping reflexes with males of this strain. Although it is conceivable that chemical stimulus is involved here, there is no evidence to support this. On the other hand, there is a possible morphological difference between virgin and previously copulated females. In the latter, both the cerci and the eighth abdominal segment appeared to be more deeply retracted, and this would make it difficult for the male to seize the cerci with his claspers. This would not account for those cases where the male actively withdrew his terminalium from the female. The work of Spielman *et al.* (1967) differed from these findings, since they were able to force-copulate previously cage-mated females.

Since most (95/100) virgin females which are 48 to 60 hours old are readily force-copulated by males of the same age, an optimum standard for evaluating the behavior of controls was established. Although 10.0% (5/50) of those females which had force-copulated 12 hours earlier did so again, it is possible that this seemingly high value is due to the fact that some of these females were not initially inseminated. More data are needed on insemination rates obtained with the forced-copulation technique.

We are grateful to Mrs. Daisy Liu for her technical assistance.

SUMMARY

1. Virgin females which are 24 hours old or less interfered with the copulatory attempts of males by kicking with their legs and emitting fluid from their ani. About 50% of these young females could be force-copulated, and 75% of these were inseminated.

2. The mean forced-coital time of young virgin females which were inseminated was 71.4 seconds. Those which were not inseminated force-copulated for a much shorter time (15.6 seconds).

3. The sexual responses of males to young virgin females were not uniform with the forced-copulation technique. Active refusal by withdrawal of the male terminalium from the female by the male was more definite with young females than with older ones.

4. Prolonged coitus was observed in about 8% of the forced-copulations with young virgin females; this phenomenon has not been reported for cage-matings nor has it been seen in nature.

5. Ninety-five of 100 virgin females force-copulated, whereas only one 3-second copulation was obtained with 100 females that had been previously cage-mated.

6. A force-copulation rate of 10% was recorded for females that had force-copulated 12 hours previously.

7. The cerci and the eighth abdominal segment appeared to be more deeply retracted in inseminated females than in virgin females.

8. While individual males varied in their sexual responses toward similar females, 52% of them definitely responded to them and 92% of these individuals copulated.

9. Using the forced-copulation technique, precoital and coital time means were 16.0 and 20.7 seconds, respectively. The age of the male did not influence these values.

10. The number of copulations by 5- and 7-day-old males was significantly higher than that by 1- and 3-day-old males.

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EFFECTS OF SEMI-STARVATION ON GROWTH AND MORPHOGENESIS DURING THE LARVAL STAGES OF A COMMON MILLIPED, NARCEUS ANNULARIS (RAF.)[1]

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The life-cycle of the Diplopoda is divided into three phases: the egg, the larva, and the adult. In *Naceus annularis* (Raf.), the common northern spirobolid milliped, the egg is enclosed in a capsule fashioned out of regurgitated leaf litter by the female at the time of egg-laying (Barber, 1915). The first two instars and at least three days of the third instar are spent within the micro-environment of the egg capsule (Berns, 1968). Following emergence from the egg-capsule, the milliped goes through at least seven additional instars before sexual maturity is attained.

In most arthropods, the passage through the larval instars is mainly related to growth in size (Wigglesworth, 1965). The morphogenetic and differentiative processes (with the exception of reproductive structures and regeneration) are mainly confined to the egg, and to the pupal stage in the holometabolous insects. In millipeds, however, passage through the larval instars is not only characterized by growth, but also by a progressive increase in the number of body segments (in *Narceus annularis,* from 7 segments in instar 2, to 51–59 segments in the adult), limbs, and ocelli. Therefore, each instar is a morphogenetic and differentiative phase.

Preliminary observations (Keeton, 1960) suggested to us that environmental factors, particularly food supply, can strongly influence, in a quantitative fashion, the developmental changes occurring in each larval instar. Consequently, we set out to determine the effect of diet on growth, segment addition, and ocelli addition at each instar, as well as the number and duration of the instars. We were particularly interested in discovering which effects of semi-starvation would carry over into the sexually mature adults, and which effects would disappear as a result of developmental compensation. We also hoped to find characteristics that would be reliable in determining the instar of larvae.

Materials and Methods

Adult females of *Narceus annularis* were collected from the field and placed in plastic boxes with a maximal supply of leaf litter for food. Nine hundred third instar larvae from eggs laid by these females were used in this study. Four hundred and fifty of these larvae were placed in 15 covered plastic boxes (12″ × 4″ × 6″), 30 animals per box, with a maximal supply of leaf litter for food. The other 450 larvae were placed in 15 boxes and were given only fecal pellets

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mixed with soil (1:1) for the first six months. After six months, these larvae were given once a month an amount of leaf litter consumable in one week; they were fed only fecal pellets the remaining three weeks of each month. This modification of the original diet was necessary to prevent excessive mortality. Both groups of animals, well-fed (indicated by + elsewhere in this paper) and semi-starved (indicated by −), were maintained in the same room, at the same temperature and humidity.

Larvae from both groups were examined in each instar for body length and width, ocelli number, number of segments, and gonopod development. Records were also kept of the duration of the instars, and the total number of instars to sexual maturity.

Results

Body length and width

Length and width measurements (to the nearest 0.1 mm.) of specimens preserved two to three weeks following molting are presented in Figures 1 and 2. Length measurements were made from the tip of the head to the tip of the anal valve; width measurements were made by measuring the diameter of the fourth body segment.

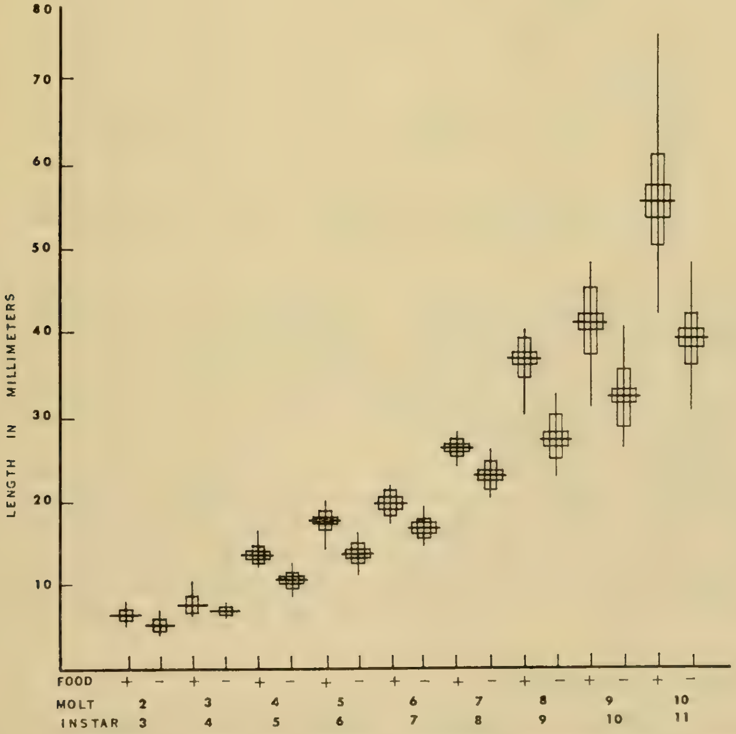

FIGURE 1. Length measurements of well-fed and semi-starved larvae.

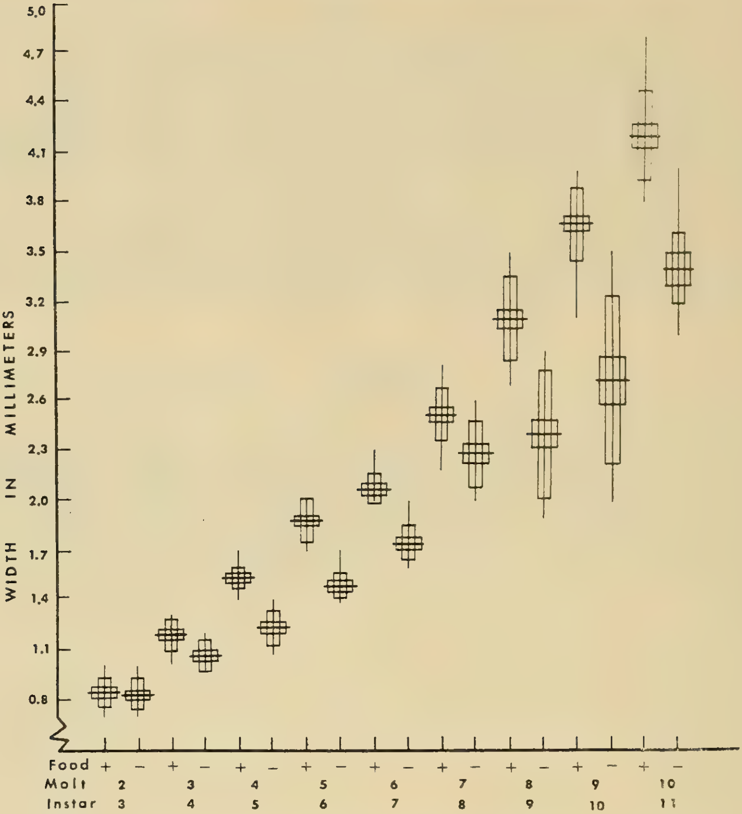

FIGURE 2. Width measurements of well-fed and semi-starved larvae.

The mean values for each instar, both length and width, are consistently lower for the semi-starved animals. In only two instars are the values not statistically different: the mean width of instars 3(+) and 3(−), and instars 4(+) and 4(−). All other means are significantly different at the p 0.001 level (t test), except for length in instars 3(+) and 3(−), and in 4(+) and 4(−), which are significant at the p 0.005 level.

It is clear that diet can affect the overall size of the animal, and therefore these two measurements (length and width) are not satisfactory indices for determining the correct instar. For example, the mean length and the distribution of length values for instar 11(−) are actually less than those for instar 10(+). The mean width of instar 9(−) is less than the mean width of instar 8(+). These are only two examples; other cases of overlap are evident in Figures 1 and 2.

Total number of body segments

The total number of body segments for both well-fed and semi-starved animals is presented in Figure 3 and Table I. The general pattern of segment increase is similar in both groups of animals. However, there appears to be a difference in the early rate of segment increase from instars 4 through 6; after instar 6 the difference between the two groups does not increase. In fact, there is a slight convergence in instars 9 and 11.

Analysis of the mean number of segments per instar was carried out using the "t" test. Comparison of the means of the two groups demonstrates a statistical difference ($p < 0.001$) in instars 5 through 11. There are instances where the mean segment number for an instar of semi-starved larvae is actually less than the mean for an earlier instar of well-fed larvae; for example, instar 11(−) is lower than 10(+). There is also considerable overlap in the ranges (Table I) of instars 9(−) and 8(+), 8(−) and 7(+), 7(−) and 6(+), and 6(−) and 5(+).

Adults of both the semi-starved and well-fed groups are within the normal range of segment number for the species (51–59; Keeton, 1960) even though the mean of the semi-starved (53.69) is less than the mean of the well-fed (54.91). The mean of the well-fed group is very close to the overall mean for the species, 55.1 (Keeton, 1960).

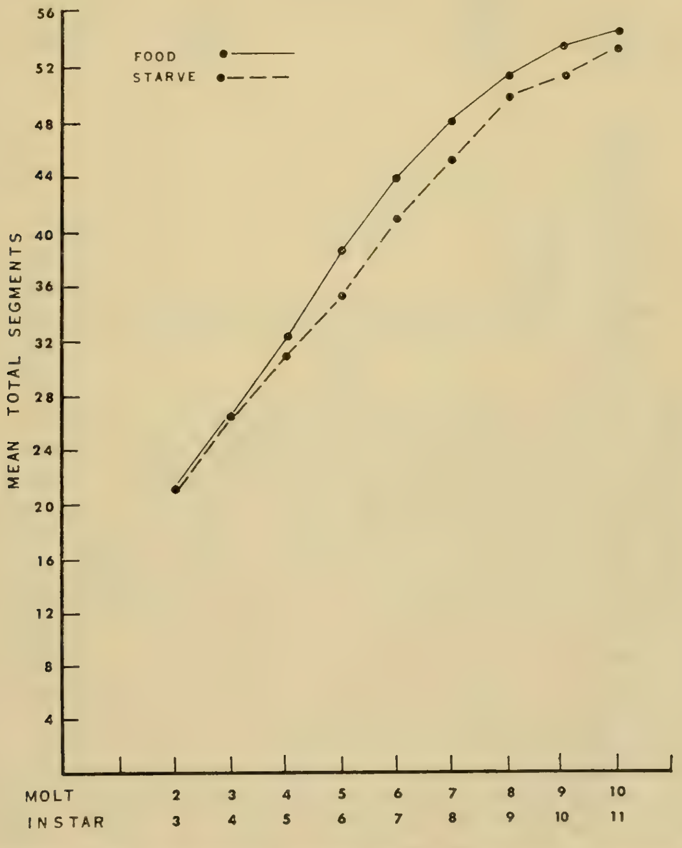

FIGURE 3. Mean total segment number of well-fed and semi-starved larvae.

Table I

Segment number per instar (well-fed +, semi-starved −)

Instar	Molt	Mean segment number	Standard deviation	Standard error mean	Range limits	N
3+	2	21.33	0.65	0.19	21–23	12
3−	2	21.00	0.0	0.0	21	13
4+	3	26.67	0.57	0.11	25–27	24
4−	3	26.54	0.80	0.16	25–28	24
5+	4	32.52	0.64	0.13	31–34	25
5−	4	30.92	0.69	0.14	30–32	24
6+	5	38.75	0.78	0.15	37–40	28
6−	5	35.40	1.10	0.25	33–37	20
7+	6	44.10	0.82	0.14	43–46	30
7−	6	41.11	1.40	0.23	38–44	35
8+	7	48.30	0.93	0.19	46–50	23
8−	7	45.52	0.92	0.18	43–48	27
9+	8	51.65	1.40	0.32	49–54	20
9−	8	50.10	1.17	0.26	48–53	19
10+	9	53.77	1.35	0.26	51–57	26
10−	9	51.65	1.74	0.43	49–54	17
11+	10	54.91	1.34	0.28	52–57	22
11−	10	53.69	1.32	0.26	51–55	26

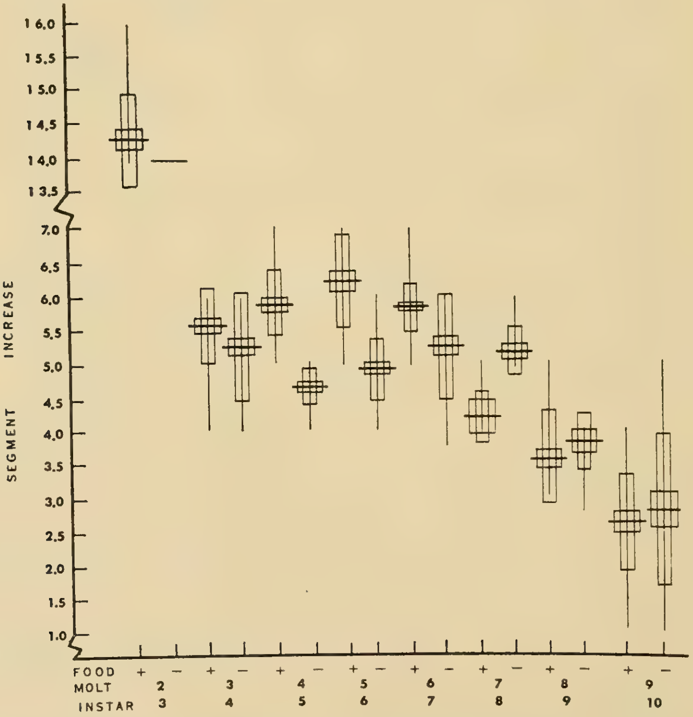

Figure 4. Segment increase per instar of well-fed and semi-starved larvae.

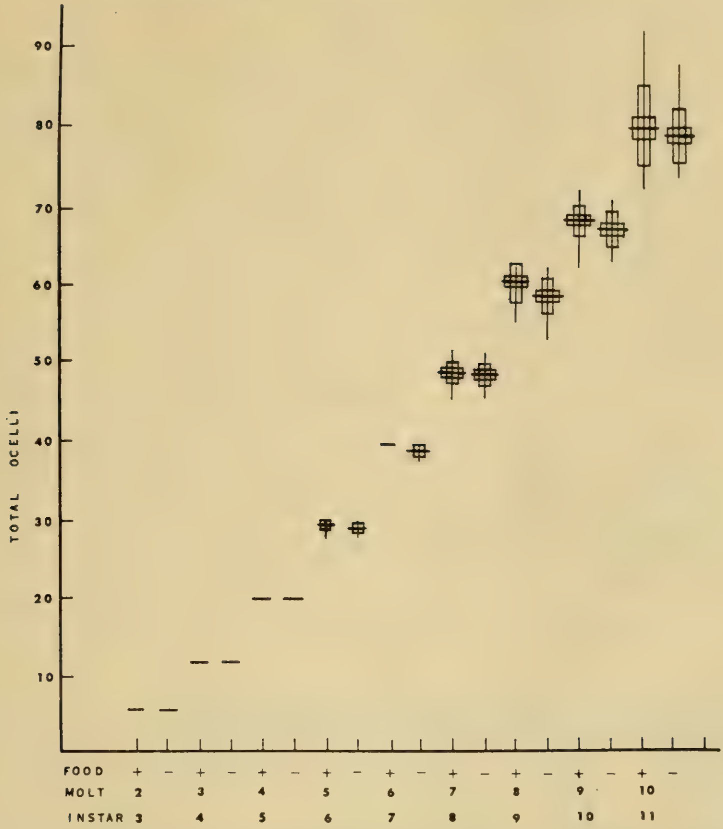

FIGURE 5. Ocelli increase per instar of well-fed and semi-starved larvae.

Segments added per instar

The effect of diet on segment addition is presented in Figure 4. At molts 4, 5, and 6, the well-fed larvae added more segments than the semi-starved (t test, $p < 0.001$). At molt 7, however, the semi-starved larvae added more segments than the well-fed ones ($p < 0.001$); the same trend was apparent at molts 8 and 9, though the difference was not statistically significant ($p < 0.3$ and 0.5).

The new segments develop from a posteriorly located proliferative region (located between the penultimate segment and the anal sgment) during the first few days of the instar preceding the molt at which they first become outwardly visible (this was confirmed histologically, and will be the topic of another paper). For example, new segments exposed by the molt (molt 4) to instar 5 actually developed during the first three days of instar 4. Consequently, since the early instar larvae are enclosed within the egg capsule until the fourth day of instar 3, dietary effects on segment addition could not be detected until the molt to instar 5; the segments added at molt 3 (from instar 3 to 4) were originally determined during the first few days of instar 3. At this time both starved and well-fed larvae were still in the egg capsule.

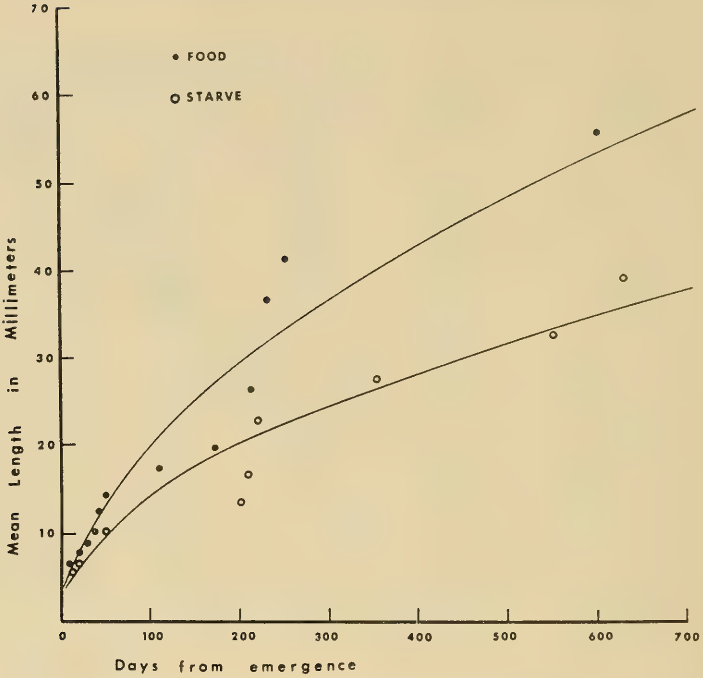

FIGURE 6. Mean body length in time of well-fed and semi-starved larvae. Each point is the mean of at least twenty animals.

Number of ocelli

Data on the number of ocelli characteristic of each instar are presented in Figure 5. In no instar is there a statistical difference between the means of the well-fed and the starved animals. In instars 3, 4, and 5, the means of the two groups are identical; the means of the remaining instars are practically the same. However, it is interesting to note that when there is a difference, the starved animals always have the lower means. Of particular significance is the lack of overlap in the ranges of the ocelli numbers for the different instars. Though the variability of ocelli number increases in the later instars, the ranges remain discrete.

A linear relationship between ocelli and number of molts is evident in Figure 5. It is not implied, however, that the correlation is between time and ocelli. On the graph, the instars are equally spaced; in reality, the time between instars is irregular and often not constant for animals in the same instar.

Number of instars

The several maturational sequences observed for *N. annularis* are shown in Table II. Though the most frequent sequence for both the well-fed and the semi-starved animals was type 3, in which there are 9 segment-building instars, two maturational instars, and maturity following the molt to instar 12, several variations were found. In particular, 25 semi-starved animals went through one

TABLE II

Maturational sequences of Narceus annularis

Sequence type	Instars					
	8	9	10	11	12	13
1.	+	−	mature			
2.	+	+	−	mature		
3.	+	+	−	−	mature	
4.	+	+	+	−	mature	
5.	+	+	+	−	−	mature
6.	+	+	+	+	−	mature

+ = segments are being constructed during this instar.
− = no segments are being constructed during this instar.

additional segment-building instar (sequences 4 or 5), and seven semi-starved animals went through two additional segment-building stages (sequence 6); no semi-starved larvae followed sequences 1 or 2. By contrast, only 3 well-fed animals went through sequence 4, and none went through sequences 5 or 6. In general, then, nutrition can affect the maturational sequence (*i.e.,* the number of segment-building instars) of the milliped.

Timing

Rearing records of a large number of larvae showed that the period from egg-laying to the end of instar 2 was always 28 days. The mean length of instar 3 was 27.5 days, and that of instar 4 was 17.8 days. Past instar 4, time is so variable that it is entirely unreliable in characterizing the instars.

In Tables III and IV, data are presented for box variability. All the animals in boxes of both semi-starved and well-fed animals were preserved at intervals, and the number of animals in each instar tabulated. Nine of ten semi-starved boxes had animals in two or more instars, and four of these nine boxes had animals in

TABLE III

Box variability of semi-starved millipeds (for each box, the number of specimens in each instar is shown).

Box no.	Days from emergence	Instar								
		3	4	5	6	7	8	9	10	11
14	20	1	24							
15	50			21						
16	185			4	16	6				
17	220				4	19	4			
18	235					6	7	3		
19	310					5	16			
20	535					1	4	14	5	
21	540							3	10	
22	560							1	9	
23	565								4	15

three or more instars. Box No. 20 had animals from instar 7 through instar 10, at 535 days from emergence. Six of 13 boxes of well-fed animals contained larvae in two instars; no boxes had animals in more than two instars. At 535 days, three animals in the well-fed group (box 12) were in instar 11, while one animal in the semi-starved group (box 20) was in instar 7. At this age, then, animals were in any of five different instars, almost half the total number of instars the animal goes through. Similarly, at about 180 days there was a four-instar spread, and equally large spreads were found for other ages.

In an attempt to get some index of development in time, data are presented plotting body length against age in days (Fig. 6). It appears that both groups (semi-starved and well-fed) have similar growth curves even though the starved are always lower than the fed. However, the periods between 100 and 250 days are very irregular. In order to make a more meaningful comparison the data have also been plotted on double-log paper (Fig. 7).

TABLE IV

Box variability of well-fed millipeds

Box no.	Days from emergence	Instar								
		3	4	5	6	7	8	9	10	11
1	20	0	24							
2	40			24						
3	110				28					
4	173					25				
5	180					5	5			
6	209						7	1		
7	212						8			
8	246						4	9		
9	253							7		
10	265							2	14	
11	533							9		
12	535								10	3
13	549								4	14

The above data are only good enough to point out trends and trend differences between the two groups. The data are not good enough to be useful in predicting size changes.

Maturation of gonopods

The male copulatory organs, the gonopods, undergo definite morphological changes as the milliped passes through the instars. Regardless of the size of the animal, the complexity of the gonopods for a given instar is always the same; it is, therefore, possible to characterize an instar by the morphology of the male copulatory organs (Berns, in press). It must be pointed out, however, that examination of the gonopods of a living larva often results in permanent injury or death. Furthermore, because of the simplicity of the female copulatory apparatus, gonopod staging is only possible in males.

DISCUSSION

From the preceding data it is clear that diet affects both the growth and morphogenetic processes that occur during the larval development of *N. annularis*. The growth effects are reflected in both length and width. However, since an effect on the number of segments is also demonstrated, one cannot fully attribute the length differences to growth alone; one might expect animals with fewer segments to be shorter. Width, however, is a good index of growth.

Diet has an effect on the number of segments built during an instar and on the number of segment-building instars. Semi-starvation results in addition of fewer than the normal number of segments in the early instars, and greater than normal number in later instars. The increase of segment-building in the later instars, even though the animals are still on a semi-starvation diet, reflects the regulatory ability of the milliped segment-building system, which is also seen in the addition of extra segment-building instars by a significant number of semi-starved animals. The result of the segment regulation, whether by increased segment-building in the later instars, or by the addition of extra segment-building instars, was that semi-starved animals achieved a total number within the normal range for the species. However, because of the effects of diet, segment number cannot be used to characterize larval instars, despite the fact that this procedure has been used by most workers in the past (Loomis, 1933; Causey, 1955; Keeton, 1960).

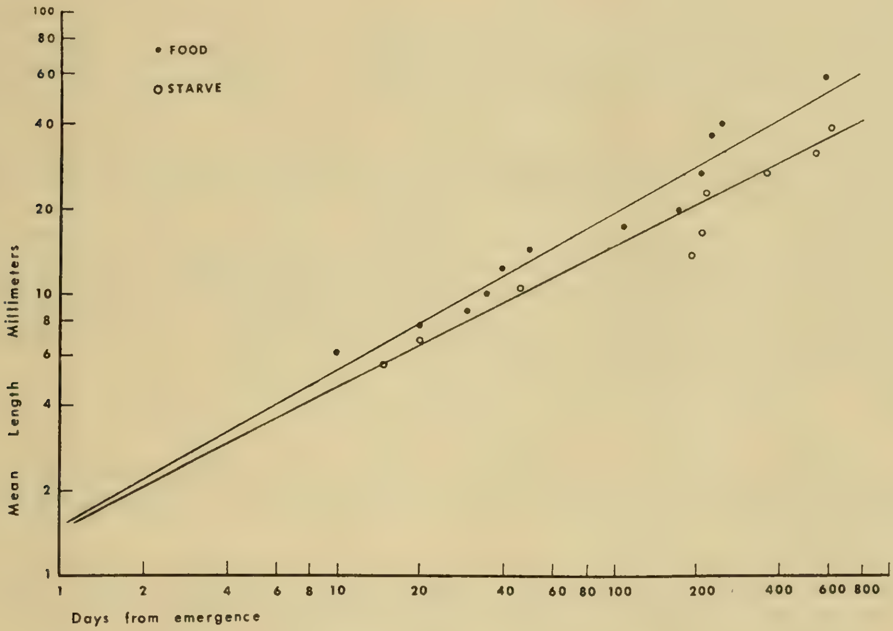

FIGURE 7. Double log plot of mean body length in time of well-fed and semi-starved larvae. These are the same data as plotted in Figure 6.

The lack of a definite number of instars is not surprising in view of similar irregularity in other arthropods, but the addition of the extra instars in the milliped appears to be related primarily to prolonging the morphogenetic process of segment-building, rather than to attaining of a required size. In fact the data demonstrate that starved millipeds mature regardless of size, as long as the segment number is within the normal range. Nutrition has been shown to influence the growth rate and the number of ecdyses of several insects: *Blatella* (Seamans and Woodruff, 1939), *Tineola* (Titschack, 1926), and *Bombyx* (Bounhiol, 1938). In these cases, however, size seems to be the critical factor for maturation.

Effects of other environmental factors on the growth and molting of arthropods are well documented (Wigglesworth, 1965). Factors such as temperature have been shown to both increase the number of molts of *Sphodromantis* (Przibram and Megusar, 1912), *Dermestes* (Kreyenberg, 1929), and *Tenebrio* (Ludwig, 1956), or decrease the number of molts in *Melanophus* and *Pieris* (Hoskins and Craig, 1935). Humidity, likewise, can effect the number of molts in *Thermobia* (Sweetman, 1934).

Not only is the number of instars variable in the milliped, but the time spent within the instars is even more variable. Semi-starvation merely exposes the limits of this variability; at 535 days of age, animals could be found in any of five different instars. Likewise, an attempt to correlate age with size was unsuccessful.

The only characteristics examined that were not significantly affected by diet were the number of ocelli and the morphological development of the male copulatory apparatus. In *Narceus annularis,* then, ocelli number is the most reliable staging characteristic in live specimens of both sexes.

Summary

1. Growth, as measured by length and width, is substantially affected by diet. The length and width measurements were significantly lower for larvae in the semi-starved group.

2. The total number of body segments was statistically lower in the semi-starved larvae in instars 5 through 11. The difference was manifest at molts 5 and 6, remained constant at molts 7 and 9, and decreased slightly at molts 8 and 10.

3. Even though the mean number of segments was lower for adults of the semi-starved group, the range of adult segment number of the semi-starved animals was the same as the well-fed group and the same as the range given for the species.

4. Starvation caused the addition of fewer than normal (as compared to the well-fed group) segments in the early instars and a greater than normal number of segments in the later instars. Similarly, a significant number of semi-starved animals passed through either one or two extra segment-building instars.

5. The time spent in instars 4 through 11 was so variable that it was impossible to establish an index of development in time. Similarly, growth in time was most variable.

6. Diet did not have a significant effect on the number of ocelli added in each instar, or on the maturation of the male copulatory organs. These two characteristics are suitable characteristics for determining the instar of larvae, though the gonopods are difficult to examine in living specimens.

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EFFECT OF JUVENILE HORMONE ON THE SYNTHESIS AND ACCUMULATION OF A SEX-LIMITED BLOOD PROTEIN IN THE POLYPHEMUS SILKMOTH

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Juvenile hormone (JH) plays a key role in insect development. As long as immature insects produce and release this agent, they do not mature. When they no longer release JH, or release it in small amount, they metamorphose. The effect of JH on the maturation of immature insects is seen when a JH extract (Williams, 1956), or pure JH (Röller *et al.*, 1967; Meyer *et. al.*, 1968) is injected into a pupa of the Polyphemus silkmoth, *Antheraea polyphemus*. When a normal pupa is allowed to develop in the absence of juvenile hormone, it molts into an adult moth about 18 days after the first signs of apolysis. However in a JH-injected pupa, adult development is blocked, and the pupa precociously molts into a so-called second pupa 10–12 days after the injection (Gilbert and Schneiderman, 1960). During the pupal-pupal molt, the insect's epidermal cells, instead of secreting adult cuticle, secrete a cuticle which closely resembles the original pupal cuticle. Also the morphogenetic death of many of the epidermal cells of the wings and antennae, which accompanies normal adult development, does not occur.

Because of its effects on metamorphosis, JH might be expected to influence the synthesis, appearance, or disappearance of specific proteins. The present report analyzes the effects of JH on the synthesis, release and accumulation of a specific protein found in the blood of female Polyphemus pupae.

MATERIALS AND METHODS

Female pupae of *Antheraea polyphemus* that had been chilled at 2° C. for six months were removed from the cold and anesthetized with carbon dioxide. Each animal was bled through a cut in the facial region between the right wing and right antenna. About 20 μl. of blood was squeezed through this cut into a 0.5-ml. polyethylene microcentrifuge tube containing a few crystals of phenylthiourea (PTU) (Schmidt and Williams, 1953). Immediately after the blood was collected, the wound was sealed with melted paraffin. The blood samples were centrifuged for two minutes in a Beckman microfuge at 12,500 g and stored at $-10°$ C.

The animals were injected with a JH extract prepared according to the method of Gilbert and Schneiderman (1960). Some of the operated animals were injected with 75 JH units/g. pupal weight (*i. e.*, 150 mg./g. pupal weight of a 1:1

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mixture of JH:peanut oil (PO)). Control animals were injected with 150 mg./g. pupal weight of PO, or were uninjected. Blood samples were collected from the same animals on consecutive days during the pupal-pupal molt and during the pupal-adult transformation. On each succeeding day, 5–10 μl. of blood was collected through the original wound. After each such operation, the wound was immediately resealed. JH-injected animals were bled every day until they molted into second pupae 8–10 days after the injection. Control animals were bled daily for 17 to 21 days. The fat body and ovaries were removed from all animals at the end of the experiment and were stored at −10° C.

The proteins in these blood samples were separated electrophoretically, and their relative concentrations were estimated by densitometry.

All samples were examined using high pH discontinuous electrophoresis as described by Davis (1964) and Ornstein (1964). Preliminary experiments, in which the gel concentrations and the amount of protein applied to each gel were varied, indicated that the desired proteins were best resolved by electrophoretically separating 0.5 μl. of blood in 4.5% gel. This quantity of blood was applied to the gel column as 10 μl. of a 1/20 solution of blood in insect Ringer solution (Ephrussi and Beadle, 1936) and separated electrophoretically.

Upper and lower gel solutions were purchased from Canal Industries, Silver Spring, Maryland. Gels were prepared according to the manufacturer's instructions. The upper and lower buffers were prepared by diluting a stock solution containing 6.0 g. Tris and 28.8 g. glycine per liter of distilled water 1:9 with distilled water.

Electrophoresis was carried out at 22–24° C. at 3 mA/gel until the bromphenol blue tracking dye reached the bottom of the lower gel, usually 50–60 minutes. Following electrophoresis, the gels were removed from their holders and stained for 30 minutes with a solution of 0.5% Buffalo Black in 7.5% acetic acid, and destained electrophoretically in 7.5% acetic acid.

The destained gels were scanned in a Joyce-Loebl densitometer at a 1:9 ratio (1 mm. of gel length = 9 mm. of record). The machine measured and recorded the density of the stained protein bands, and automatically integrated the area under the curve for each band. The densitometric data for Polyphemus blood proteins were compared with standard densitometric curves obtained with specific amounts of beef serum albumin, and the amount of protein in a given band was expressed as μg. of beef serum albumin equivalents (N. G. Patel, unpublished observations). These measurements were accurate to ±5%. The blood collected from three experimental animals and three control animals was analyzed in this way.

The fat body and ovaries of experimental and control animals were thawed, suspended in cold Ringer solution in microcentrifuge tubes containing PTU, and sonicated for five minutes in a Bronwill cup sonicator at maximum probe intensity. Microscopic examination revealed that this treatment ruptured all of the cells. The samples were then centrifuged at 12,500 *g* for five minutes. The low density fatty layer was skimmed from the top of each sample, and the supernatants were then separated electrophoretically.

In some experiments, fat body from female Polyphemus pupae was implanted into the abdomens of male pupae, which were allowed to develop into adults. Blood was collected from these animals, and analyzed electrophoretically.

RESULTS

1. *Effects of various treatments on development*

The blood sampling procedure had no effect on development. Both experimental and control animals showed the first visible signs of development of apolysis two days after removal from the cold. Control animals completed their metamorphosis 20–22 days after removal from the cold, whereas experimental animals completed their pupal-pupal development 8–10 days after removal from the cold.

The second pupae were covered with a pupal cuticle. Their ovaries showed no signs of development, and as far as could be determined were morphologically and histologically pupal. In contrast, the ovaries of control animals had differentiated in the manner described by King and Aggarwal (1965) although none of the eggs were covered with a chorion.

The fat body of second pupae also appeared pupal by morphological criteria. The cells of pupal fat body are held together in sheets by a connective tissue sheath. Early in adult development, this sheath breaks down and most of the fat body cells become loosened from each other. Later in adult development, the sheath reappears, and the fat body cells are again held together, this time in strands (Madhavan and Schneiderman, 1968). This sheath failed to break down in JH-injected pupae, and was retained when pupae molted into second pupae.

2. *Proteins detected in the blood of female Polyphemus pupae*

Figure 1 illustrates the protein bands that can be detected in the blood of a normal female Polyphemus pupa freshly removed from the cold. In addition to many lighter bands, whose visibility can be increased by applying larger amounts of protein and by varying the conditions of separation, the electrophoretic pattern of female blood contains two conspicuous protein bands. These are designated 4B (R_f with respect to the tracking dye = 0.47 in 4.5% gels) and 5A (R_f with respect to the tracking dye = 0.51 in 4.5% gels). Bands 4B and 5A were not resolved further in experiments which varied the gel concentrations and the amount of protein applied to each gel. For this reason, each band is thought to represent a single species of protein molecule. As seen in Figure 1, protein 4B is sex-limited. It probably corresponds to the sex-limited yolk protein antigen first described by Telfer and Williams (1953) in *Hyalophora cecropia* (their "antigen 7"). Protein 5A is not sex-limited.

3. *Changes in the concentrations of proteins 4B and 5A during development*

Densitometric determinations were made on the blood samples collected from three experimental animals and three control animals and expressed as μg. beef serum albumin equivalents. They are plotted in Figure 2, and reflect the changes in concentration of protein bands 4B and 5A. As Figure 2 shows, the concentration of protein 4B is markedly affected by juvenile hormone whereas the concentration of protein 5A is very little affected. In JH-treated pupae protein 4B may reach twice the concentration it has in control animals. Figure 3 records the ratio (protein 4B)/(protein 5A) and demonstrates that this ratio differs in experimentals and controls largely because of the changing concentration of protein 4B.

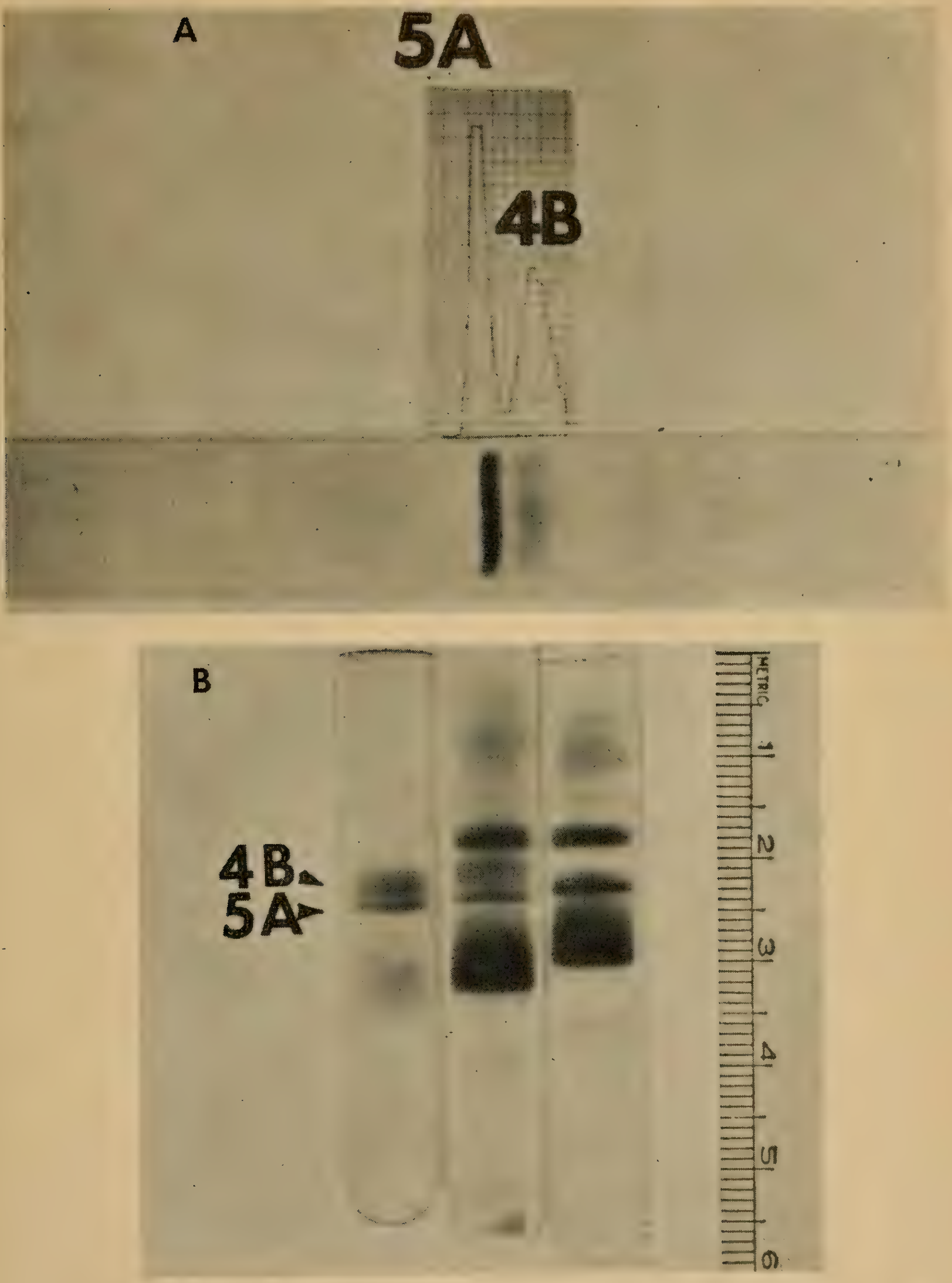

FIGURE 1. Electrophoretic separation of pupal Polyphemus blood in 4.5% acrylamide gels. A. Electrophoretic separation and densitometric recording of bands 4B and 5A in female blood. The densitometric values obtained were converted to μg. of beef serum albumin-equivalent by referring to a standard curve. B. Left to right: 0.5 μl. of female blood; 0.5 μl. of female blood + 2.0 μl. of male blood; 2.0 μl. of male blood. Band 4B is not visible in male blood.

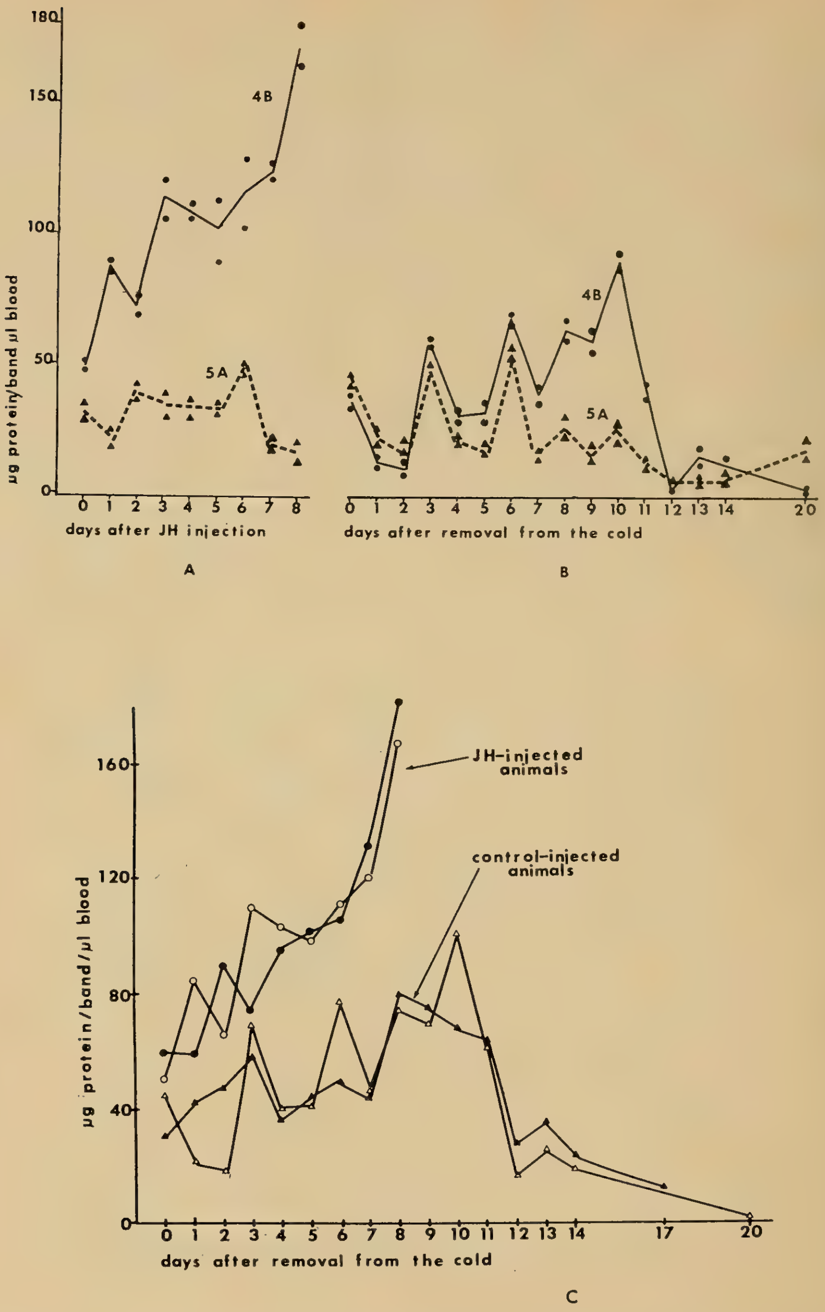

FIGURE 2. Changes in the concentrations of protein bands 4B and 5A in the blood of females during adult and second pupal development. A. Daily measurements of the concentrations of bands 4B and 5A in a typical animal injected with JH. Each point is based

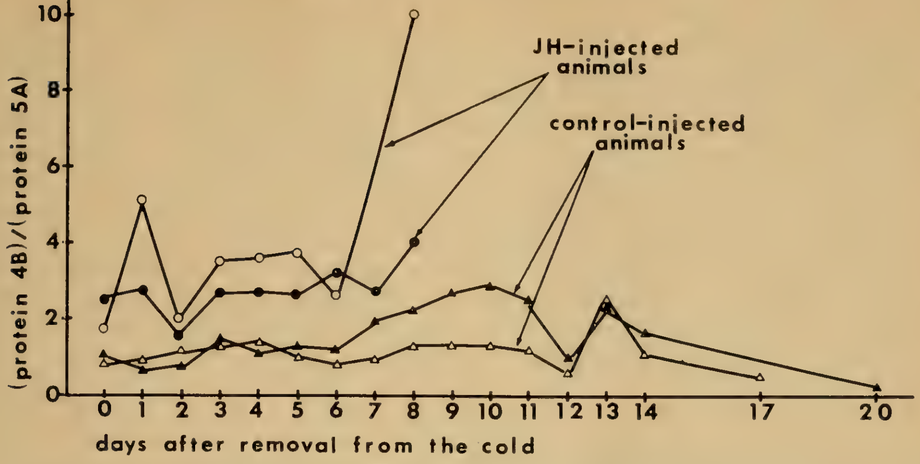

FIGURE 3. Comparison of the daily changes in the ratio of (protein 4B)/(protein 5A) in the blood of JH-injected and control animals. Each line represents one animal. Each point represents the average of the ratios of 4B to 5A.

4. *Proteins in the fat body and ovaries*

The results of electrophoretic separations of the protein in the fat body and ovaries of experimental animals are summarized in Tables I and II, and indicate the following:

1. The ovaries of adults contain proteins 4B and 5A. The ovaries of pupae and of JH-injected second pupae, in contrast, contain protein 5A, but do not contain protein 4B.

2. The blood of female pupae, 0–17-day developing adults, and JH-injected second pupae contains proteins 4B and 5A. The blood of adult females contains protein 5A, but does not contain protein 4B.

TABLE I

The distribution of protein bands 4B and 5A in the blood, fat body and ovaries of female pupae, developing adults, adults, and JH-injected second pupae

Band number	R_f in 4.5% gel	Blood				Fat body			Ovaries		
		P	DA	A	P_2	P	A	P_2	P	A	P_2
4B	0.47	+	+	0	+	0	0	0	0	+	0
5A	0.51	+	+	+	+	+	+	+	+	+	+

P = pupa; DA = 0-17-day developing adult; A = adult; P_2 = second pupa; + = present; O = absent.

on densitometric measurements of one or two separate gels. B. Daily measurements of the concentrations of bands 4B and 5A in a typical control animal. Each point is based on densitometric measurements of one or two separate gels. C. Comparison of the daily changes in the concentrations of protein 4B in the blood of JH-injected and control animals. Each line represents one animal. Each point represents the average of densitometric measurements of two or more separate gels.

3. The fat body of males and females at all stages contains protein 5A, but does not contain protein 4B.

The observation that the female fat body does not contain protein 4B prompted further experiments to establish whether the fat body synthesizes and then releases protein 4B, as has been reported for other blood protein (see Discussion). Telfer (personal communication) has obtained immunological evidence that the fat body of Cecropia synthesizes and then releases the yolk protein, antigen 7. To confirm Telfer's observations, female fat body was implanted into the abdomens of male pupae, which were then allowed to develop into adults. Protein 4B was detected in the blood of these developing adults, whereas in normal developing male adults, protein 4B was never detected (Table II). Efforts to detect the synthesis and release of protein 4B by fat body incubated *in vitro* in blood and various media failed (Blumenfeld, 1968).

Table II

The distribution of protein bands 4B and 5A in the blood and fat body of male pupae, adults, and JH-injected second pupae

Band number	R_f in 4.5% gel	Blood				Fat body			
		P	A	A_f	P_2	P	A	A_f	P_2
4B	0.47	0	0	+	0	0	0	0	0
5A	0.51	+	+	+	+	+	+	+	+

P = pupa; A = adult; A_f = adult derived from male pupa which had received an implant of female pupal fat body; P_2 = second pupa; + = present; O = absent.

Discussion

1. *The fate of protein 4B*

The behavior of blood proteins in Polyphemus is best considered in relation to the behavior of the blood proteins of a closely related saturniid silkmoth, the Cecropia silkworm, which have been the subject of extensive experimental analysis. The blood of the female Cecropia pupa contains a high concentration of a specific protein antigen that is present in very low concentration in the blood of the male pupa (Telfer and Williams, 1953; Telfer, 1954). This antigen, which they called "antigen 7," is accumulated by the developing oocytes to a concentration above its level in the blood, and disappears from the blood during metamorphosis. During this time, several other blood protein antigens that appear in the oocytes are not accumulated to concentrations above their level in the blood. Antigen 7 corresponds to an electrophoretically separable protein band (Laufer, 1960). Proteins that correspond to antigen 7 are present in the blood of other saturniid moths (Laufer, 1960). Protein 4B discussed in these experiments is sex-limited, is accumulated by the developing oocytes, and appears to correspond to antigen 7.

At most stages of adult development, a change in the concentration of protein 4B is paralleled by a change in the concentration of protein 5A. Between 0 and 5 days of adult development, the concentrations of proteins 4B and 5A varied from 30 to 50 $\mu g./\mu l$. Between 5 and 7 days of adult development, protein 5A attained a concentration as high as 65 μg./μl., while protein 4B attained a concentration in the blood of 100 μg./μl. The concentrations of both proteins then decreased as the accelerating demands of the maturing oocytes removed them from circulation. By 10 days of adult development, protein 5A had fallen to 35 μg./μl., while protein 4B had fallen to 25 μg./μl. After this time, they decreased further in concentration.

When one injects JH into the female pupa, one discovers that JH completely inhibits ovarian development, and that second pupal ovaries are morphologically and histologically indistinguishable from those of pupae. In most insects JH either directly or indirectly exerts a trophic effect on the oocytes and is required for both oocyte growth (see review by de Wilde, 1964) and yolk accumulation (see reviews by Telfer, 1965; Engelmann, 1968). Since extirpation of the pupal corpora allata has no obvious effect on the egg development of many non-feeding higher Lepidoptera (Williams, 1959) it is generally believed that the presence of JH during adult development is not necessary for egg development in this group. In these insects egg development occurs during adult differentiation and appears to be coupled to it. Thus, when one blocks adult differentiation with JH, one perforce blocks egg development.

Changes in the blood levels of proteins 4B and 5A are closely linked during normal adult development. When one injects JH, however, this relationship is uncoupled, and the concentrations of the two proteins diverge. In the present experiments this uncoupling was first detected seven days after JH injection. The JH injection results in a 3–4-fold increase in the titre of protein 4B from 40 to about 180 μg./μl. 8 days after injection. During this time, protein 5A remains within its normal range. Telfer (1954) ovariectomized female Cecropia pupae, and found that the level of the yolk protein in the blood of such animals rose to more than twice its level in the diapausing pupa. The rising titre of protein 4B in JH-injected female Polyphemus pupae strikingly parallels Telfer's results. Indeed, as far as protein 4B is concerned, JH appears to produce the same effects as ovariectomy.

The career of protein 4B consists of periods of synthesis, release into the blood, and accumulation by the growing oocytes. JH does not affect the synthesis or release of protein 4B, but specifically blocks the accumulation of protein 4B by the oocytes. Thus, JH profoundly upsets the career of protein 4B.

2. *What tissue synthesizes and releases protein 4B?*

While there is conclusive evidence that the fat body synthesizes and releases specific blood proteins (Shigematsu, 1958; Price and Bosman, 1966) it has not been proven that the fat body synthesizes and releases the yolk protein of the female. Telfer (personal communication) has concluded, from immunological analyses, that the female fat body of Cecropia was indeed the source of this protein. In the present experiments, the appearance of the yolk protein, protein 4B, in the blood of males that had received implants of female fat body confirms Telfer's findings.

JH injection prevents the breakdown of the connective tissue sheath around the fat body, but does not block the synthesis of protein 4B by the fat body cells themselves. Hence, the data suggest that as far as the synthesis of this particular protein is concerned the pupal fat body is not a biochemical target of JH at the concentrations we employed. There is also other evidence that JH injection does not prevent an adult-specific protein from appearing in the fat body (Blumenfeld, 1968).

One wonders why the biochemical metamorphosis of the pupal fat body is not blocked by juvenile hormone whereas the metamorphosis of the integument is so profoundly affected. The answer may stem from the fact that many insect cells appear to be most sensitive to juvenile hormone when they are about to synthesize or are synthesizing DNA (*cf.* for example Krishnakumaran *et al.*, 1967, p. 43–44 ff.). Pupal fat body engages in very little DNA synthesis during the pupal-adult transformation whereas epidermal cells do engage in DNA synthesis, hence the insensitivity of pupal fat body to juvenile hormone.

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Summary

Injection with juvenile hormone causes pupae of the Polyphemus silkmoth to undergo a pupal-pupal molt instead of a pupal-adult molt. Juvenile hormone also affects the concentration in the blood of a sex-limited blood protein, the yolk protein, which is normally synthesized by the fat body of the female pupa, released into the blood, and accumulated from the blood by the developing oocytes. When juvenile hormone is injected into a female pupa, the synthesis and release of the yolk protein are not affected. However, the oocytes do not grow and do not accumulate the yolk protein. Consequently, the concentration of the yolk protein in the blood of developing second pupae increases about four-fold. This effect becomes conspicuous seven days after juvenile hormone injection.

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LIGHT-INHIBITION OF FLASHING IN THE FIREFLY PHOTURIS MISSOURIENSIS[1]

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Recent investigations of photic inhibition of flashing in the firefly *Luciola* (Magni, 1967; Brunelli *et al.*, 1968a, 1968b) have confirmed the phenomenon of inhibition *via* the visual system reported by Case and Buck (1963), who suggested that the observed inhibition was largely of central origin although they did observe, under conditions of continuous glowing, "unflashes"—dimming of continuous glowing with flash kinetics—upon electrical excitation of the light organ, raising the possibility of a peripheral mechanism. In *Luciola,* however, there appears to be a possibly humorally linked inhibition somehow involving the testis, as evidenced by transferral of inhibition across a saline bridge from a light-inhibited firefly and by disappearance of this phenomenon upon castration of the light-inhibited individual. The inhibitory process in *Luciola* was further shown to have a peripheral element by inhibition of flashes driven by electrical stimulation of the light organ upon illumination of the eyes (Magni, 1967).

Since peripheral inhibition has not readily been demonstrated in insects it is most desirable to determine the extent to which the *Luciola* inhibitory system is present in other species of fireflies.

The present report represents an unsuccessful attempt to detect peripheral inhibition in *Photuris missouriensis.* It provides additional information regarding the nature of the inhibition, originally reported in *Photuris* by Case and Buck (1963), and which seems to be wholly a central nervous system process acting on an exclusively excitatory peripheral light organ innervation.

MATERIALS AND METHODS

Fireflies were obtained principally from collectors in Kansas and Iowa, for whose supervision we are most indebted to Dr. Katherine Smalley of Kansas State College, Emporia. Until use, the fireflies were fed sucrose solution and kept at room temperature or stored at 10° C. Luminescence was detected *via* fiber optic light guides and 931A photomultipliers and recorded on an Offner Dynograph and photographically from a Tektronix 533 oscilloscope. Electrical excitation was delivered from constant-current stimulators or pulse generators. Light stimuli were delivered *via* a fiber optic guide from an incandescent or xenon arc lamp.

Localization of light stimuli was insured by mounting insects with their heads projecting into a hole in a black plastic block receiving the stimulating light guide and sealing the aperture with a mixture of powered graphite in Vaseline. Light

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TABLE I

Time required to photically inhibit spontaneous flashing

Illumination lead time (msec.)	Extent of inhibition of expected following flashes (1.0 = normal; 0.0 = no flash)					
0–40	1.0	1.0	1.0	1.0	1.0	
41–80	1.0	1.0	1.0			
81–120	1.0	0.6	1.0	1.0	0.8	
121–160	0.6	0.0	1.0	0.0	0.2	1.0
161–200	0.0	0.0	0.0	0.0		
201–240	0.0	0.0	0.0	0.0	0.0	0.0
241–280	0.0	0.0	0.0			
281–960	24 expected flashes, all 0.0					

Illumination 3800 lux. Combined data collected from 3 male *Photuris* by random illumination during sequences of regular spontaneous flashing.

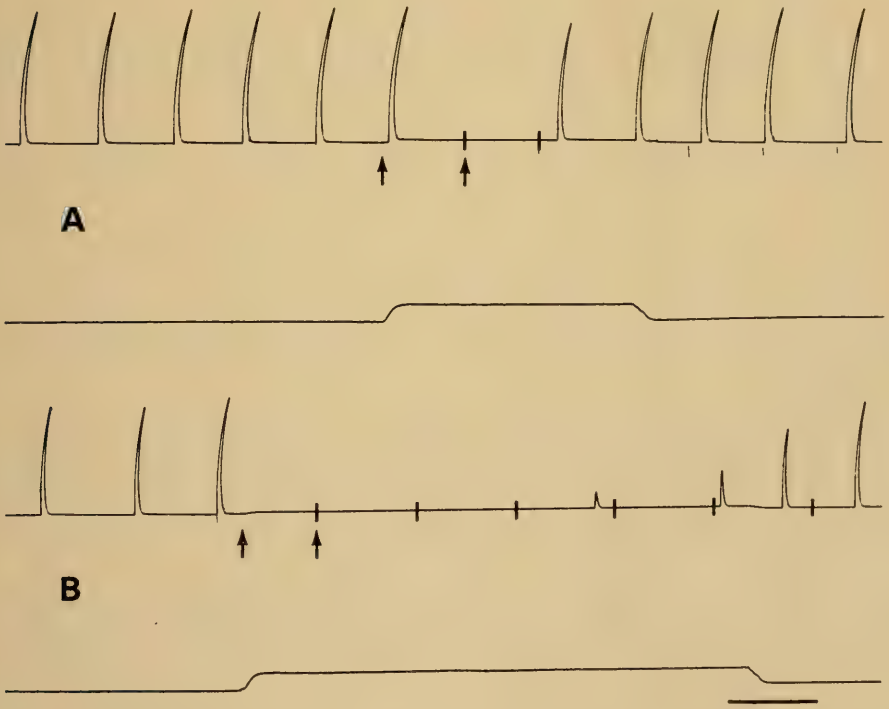

FIGURE 1. Example of records used in determination of latency of photic inhibition *via* eyes of spontaneous flashing *Photuris*. Upper trace in each record registers flashes and lower monitors light source. Latency, the interval between arrows, is measured as time between start of illumination and time of next succeeding expected flash, indicated as vertical bars, as measured from inter-flash interval of several preceding flashes. In 1A, the light goes on insufficiently early to inhibit one flash, completely inhibits the next and possibly partially inhibits and delays a third. Better examples of partial inhibition are shown in 1B. Light stimulus 100 lux, time mark at lower right, 1 second.

sources were calibrated against a Gamma Instruments standard lamp using a Reeder Thermopile and Keithley millimicrovoltmeter. Nor-epinephrine was injected with a Hamilton microliter syringe. The saline of Brunelli *et al.* (1968a) was used for preparing nor-epinephrine solutions and in the saline bridge experiments.

RESULTS

1. *Inhibition of spontaneous flashing*

Interruption of spontaneous flashing by electrical stimulation of the eye (Case and Buck, 1963) or by illumination of the eye (Magni, 1967) was readily confirmed in *Photuris missouriensis*. In this species the specificity of the interruption appears to be sufficient to permit use of the term inhibition in regard to the phenomenon. Certainly, the effect is not simply an alarm reaction since *Photuris* responds to injury or disturbance with rapid flashing.

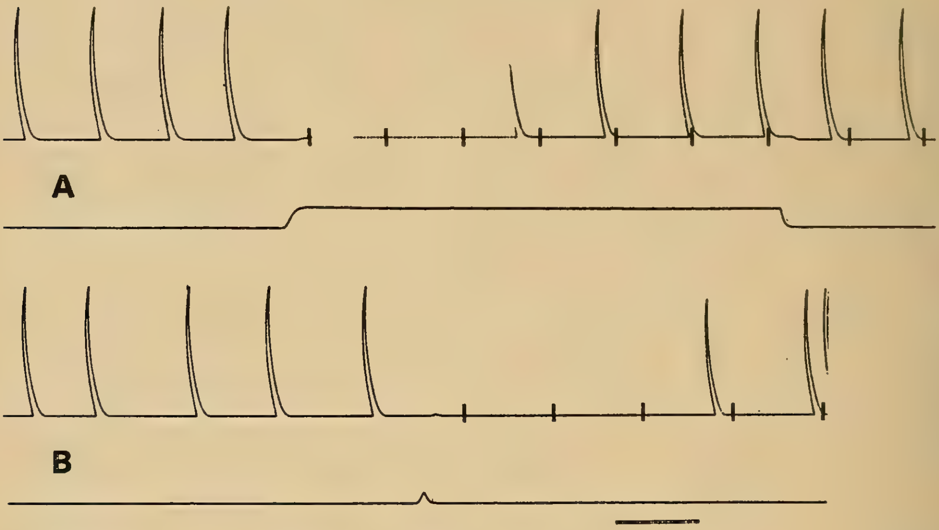

FIGURE 2. Two sequential photic inhibitions showing that a very brief ⅕-second, 50-lux flash (2B) can be as effective as a much longer, 100-lux illumination. Upper trace, light organ with some registration of stimulating lamp, which is recorded on lower trace. Time mark, 1 second.

The latency of inhibition is nearly the same for electrical and photic stimulation of the eye. Our most extensive latency measurements were made with light stimuli from either an incandescent or xenon arc lamp. Light stimuli were delivered at random during episodes of regular spontaneous flashing. Latency was estimated as the time from initiation of illumination to the time of the next succeeding modified flash (usually reduced in magnitude) or completely suppressed flash (time of expected occurrence was estimated from the average interflash interval of the preceding series). Results appear in Table I and an example of the records from which the Table was constructed appears as Figure 1. Clearly, illumination at intervals as brief as 160 msec. prior to an expected flash is completely inhibitory. It is possible to attain inhibition with a lead time of 120 msec. Lead times less than 80 msec. do not inhibit the immediately subsequent flash while they do inhibit the next and following flashes. These observations are not unexpected since Case and Buck (1963) have shown the minimum latency for excitation of luminescence by brain stimulation in *Photuris* to be 120–150 msec.

2. *Effects of light intensity and duration on inhibition of spontaneous flashes*

These effects are most puzzling since there appears to be a triggering effect of light superimposed upon a graded response. Triggering is illustrated in Figure 2 where an extremely brief illumination of not more than $\frac{1}{5}$th second with maximum intensity of 50 lux resulted in as much inhibition as continuous illumination of 100 lux for six seconds. Light-dark transitions appear often to be more significant than the total illumination delivered because some *Photuris* can be maintained in the inhibited state for a longer period by a train of approximately one second on, one second off illuminations than by continuous illumination.

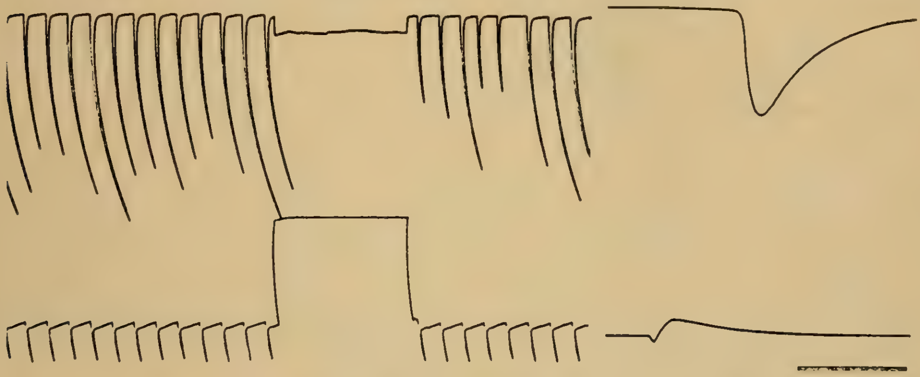

FIGURE 3. Electrical stimulation with paired electrodes in anterior light organ. Flashes recorded as downward deflection of upper trace. Stimulus (5 msec., 5 volts, 1 per second) artifacts and light (1000 lux) on lower trace. Right hand record indicates excitation-flash latency of 136 msec. Time mark at lower right applies to both records: 5 seconds, left; 200 msec. right.

Almost invariably, during all except the most intense illuminations, flashing is reestablished. Typically (Fig. 1b) these escapes are of lower than normal intensity and at a slower frequency than prior to illumination. (The kinetics of the flashes are normal.) There is a marked tendency, however, for flashes subsequent to the first several escapes to occur at the same frequency as prior to illumination and at the same expected time as estimated from pre-illumination flashes, suggesting that illumination does not inhibit a flash pace-making mechanism.

3. *Attempts at inhibition of driven flashes*

Inhibition of flashes driven at the level of the light organ were unsuccessful in all instances in which the driven flashes were without CNS augmentation. An example of what would appear to be such inhibition is Figure 3 in which a long series of flashes generated in register with 1/sec. stimulation of the light organ are terminated by illumination. However, the excitation-flash latency (measured oscilloscopically) in this sequence amounts to 136 msec. While this is probably insufficient time to allow facilitative effects *via* the brain, there is clearly sufficient time for reflexive facilitation *via* the abdominal ventral nerve cord. Even if the excitation-flash latency were sufficiently short to preclude CNS effects on the immediately following flash, there is no assurance that a long-term facilatory state built up in the CNS does not affect the flashing sequence. This is illustrated in Figure 4 where a series of voluntary flashes is mixed with driven flashes. Immediate inhibition of the voluntary component occurs upon illumination, along with a gradual diminution of the driven component. We attribute this to an effect of light on the voluntary component, defacilitating the driven flashes. This seems to be confirmed by the events after cessation of illumination in this same experiment in which the driven flashes remain at low ebb until spontaneous flashing resumes. If the level of excitation had been somewhat lower, we presume that the experimental record would have created the impression of complete inhibition of driven flashes by illumination.

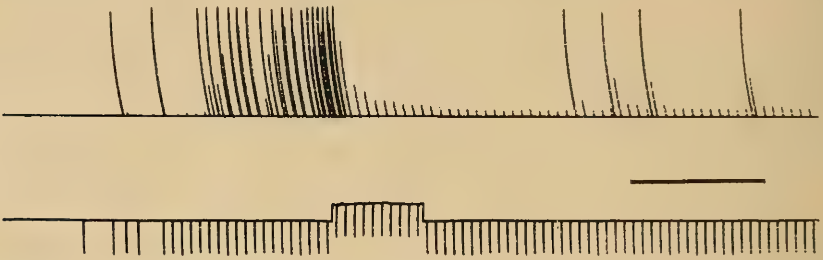

FIGURE 4. Effects of light on voluntary and driven flashes. A series of voluntary flashes, large flashes of uniform intensity, occurs along with electrically driven flashes of irregular intensity. Intensity of driven flashes is seen to depend upon temporal proximity to a previous voluntary flash. Illumination terminates voluntary component with progressive consequent diminution of driven flash magnitude. Upper trace, light organ. Lower trace, stimulus artifact (downward) and illumination artifact (upward). Time mark, 30 secs.

4. *Attempts to effect transfer of inhibition from one firefly to another*

The experimental arrangement used by Brunelli *et al.* (1968a) to demonstrate inhibition transfer between individuals in pairs of *Luciola italica* was duplicated in all essential particulars using males of *Photuris missouriensis*. *Photuris* was used in preference to other American species of fireflies because it most readily flashes spontaneously under experimental conditions. In five experiments with good spontaneous flashing it was not possible to demonstrate transfer of inhibition of spontaneous flashing from one *P. missouriensis* to another upon illumination of the eyes of one animal with light (Fig. 5). Since inhibition transfer occurred in the *Luciola* experiments in 8–15 seconds, we attempted to obtain an estimate of the minimal transfer time of an appropriate chemical agent from one insect to another in our experimental arrangement. Nor-epinephrine was chosen because it rapidly induces glowing and is probably related to the normal synaptic mediator of the firefly light organ (Smalley, 1965). The time required for transfer was measured as the time between induction of glowing, measured with photomultipliers, in one nor-epinephrine-injected member of a saline-linked pair and its non-injected mate. Even where two insects were in direct physical contact *via* the wounds produced by complete severance of their terminal abdominal segments and when one member of the pair had received an injection of nor-epinephrine sufficiently large to induce instantaneous glowing in its own light organ (8 μl. of 10^{-2} M), the minimum transfer time was 68 seconds. One of these experiments is illustrated in Figure 6. In another series of experiments, nor-epinephrine was introduced into a drop of saline in contact with the hemocoel of the second light organ segment and the time for initiation of glowing in the adjacent segment of the same animal was determined. In these experiments the minimum time required was 30 seconds. Twice this time would, we believe, be comparable to the transfer time between a pair of insects and does, indeed, correspond well with the transfer experiments just described.

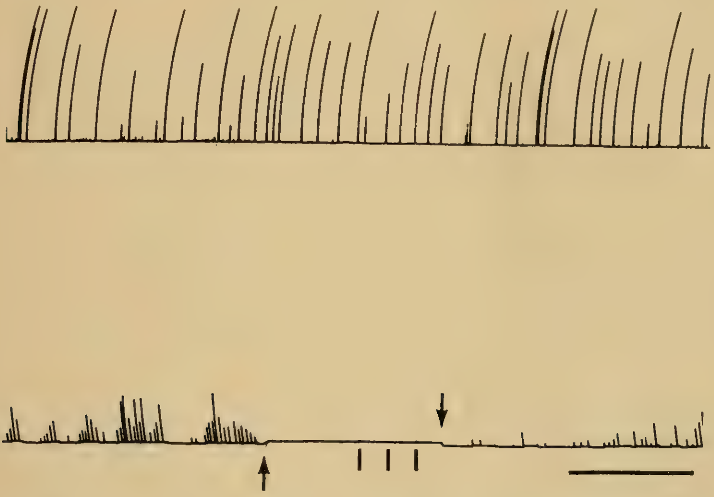

FIGURE 5. Record of an unsuccessful attempt to transfer inhibition from one light-inhibited *Photuris* male, lower trace to another, upper trace, *via* a saline bridge (see text). Light on and off indicated by arrows, 1000 lux. Three minimal escapes, indicated by vertical bars, confirm completeness of inhibition as does the long time required after cessation of illumination to reestablish rapid flashing. Upper trace shows no indication of transfer. Time mark, 30 sec.

5. *The role of the testes in flash inhibition*

Brunelli *et al.* (1968b) report that castration of the light-exposed member of a saline-linked pair made it more difficult to light-inhibit the castrated insect and made transfer of inhibition to the intact member of the pair impossible. We attempted to further assess the role of the testis by electrical stimulation of a freshly excised pair of *Photuris* testes in a saline drop directly in contact with the light organ tissue of a spontaneously flashing male *Photuris*. Inhibition of flashing was not observed.

Next a more general experiment was conducted to determine if any structures other than CNS and light organ were involved in photic inhibition. The light organ of a spontaneously flashing male *Photuris* was dissected in such a way that its only connection with the remainder of the animal was by way of the ventral nerve cord. In this state, photic inhibition of spontaneous flashing was still obtainable.

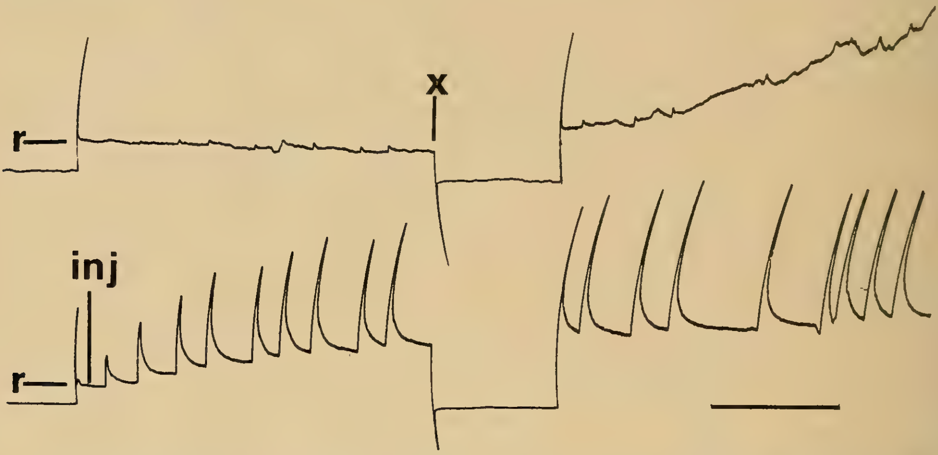

FIGURE 6. Experiment to measure diffusion time of nor-epinephrine between light organs of two fireflies with body cavities joined (see text). Lower trace records rise of luminescence, superimposed upon spontaneous flashing, upon injection at mark from rest luminescent level, r. Upper trace records luminescence from the other member of the pair. Beginning at x, the photomultipliers were turned off during visual confirmation of integrity of saline linkage. By the time recording resumed the second animal had commenced to glow, indicating arrival of nor-epinephrine and leaving the interval terminated at x, 80 seconds, as the minimum diffusion time. Time mark, 30 sec.

DISCUSSION

These data, along with the observations of Case and Buck (1963), Magni (1967) and Brunelli *et al.* (1968a, 1968b), are unfortunately subject to more than one interpretation. When one especially considers the evidence of Carlson (1961) that neural activity exerts a trophic as well as excitatory effect on the lantern, together with the observations concerning the difficulty of "arousing" flashing behavior in fireflies during the day (Case and Buck, 1963), it would seem plausible to propose two flash-suppressing phenomena, both of which are light-initiated. One of these might regulate the circadian cycle of luminescence known to occur at least in *Photinus* (Buck, 1937). The other might effect rapid control of luminescence in response to irregular, brief illuminations during the normally active period. It is plausible to suppose the former might involve a neuro-endocrine link, such as has frequently been observed in insect circadian rhythms while the latter, because of its rapidity of establishment and of dissipation, might be supposed to be wholly neural in mechanism. The phenomena described in *Luciola* strongly suggest that both mechanisms are present. However, in *Photuris* there does not at present appear to be evidence for any mechanism except the exclusively neural one. In fact, even such phenomena as the "arousal" process and Carlsons' trophic effect might well be simply due to either diminishment of excitation or to a dark-dependent neural excitatory process, rather than peripheral inhibition.

Quenching phenomena, such as described by Case and Buck (1963) have been suggested as possible examples of perhipheral inhibition of steady glowing. Since the mechanism of luminescence control *in vivo* remains unexplained, there is little restriction upon the elaboration of theories concerning peripheral processes such as this electrically driven quenching of denervated light organ glows. By analogy with other bioelectrically activated systems (see, for example, Eckert, 1966) it is plausible that light emission is related to depolarization of photocytes and this concept is strengthened by the K^+ dependence of scintillation in *Photuris* (Carlson, 1967). In glowing light organs, such as those showing the quenching effect, it might be assumed that most photocytes are to some extent depolarized. Electrical excitation of such populations with bipolar electrodes would then probably have hyperpolarizing effects in the vicinity of the anode, resulting in quenching, while cathodal effects would be minimal or non-existent, owing to the already depolarized state of the photocytes.

Resolution of the differences which appear in flash inhibitory mechanisms in *Luciola* and *Photuris* is not readily achieved. Certainly unequivocal photic inhibition of flashes driven at the level of the light organ has not yet been attained in *Photuris*. Their reported occurrence in *Luciola* may well represent a fundamental difference in light organ control in the two species. However. a possibly serious restriction to such interpretation is based on the difficulty of eliminating CNS-mediated facilitatory effects, these assuredly being inhibited by light. In the investigation of *Luciola,* this possibility was eliminated from consideration because the maximal excitatory facilitation time (driven flashes in deganglionated light organs) which is observed is 300 msec. (Buonamici and Magni, 1967, p. 332). Most likely, however, what is critical in experiments on insects with intact CNS and normal sensory input, such as specifically concerns us here, is the central excitatory state which may well remain elevated upon excitation for long periods of time. Thus Dethier *et al.* (1965) report persistence of elevations in central excitatory state of *Phormia*, in response to chemosensory input, of as long as 10 minutes. Such may contribute a sufficient level of facilitation to give false appearance of exclusively directly driven flashes in response to light organ stimulation. Abolishment of central contributions during illumination might then give the appearance of photic inhibition of peripherally driven flashing when, in actuality, the only effect might be simply a reduction in the total level of light organ excitation.

Persistence of photogenic volleys in the ventral nerve cord and light organ of *Luciola* is considered to be an argument for the existence of an inhibitory system acting peripherally to the brain (Magni, 1967). While photogenic volleys have not been recorded in our experiments, the reappearance of flashes in time with pre-inhibited flashes after and during photic inhibition is evidence that these volleys continue to be generated, or at least that the pacemaker producing them continues to function. The difficulty with assessing the significance of photogenic volley persistence during inhibition lies, of course, in the possibility that their effectiveness in producing flashes may well depend upon other CNS-generated excitatory activity which is not recorded. Certainly the low resolution of all recordings so far made of neural activity associated with flashing does not preclude the presence of small fiber tonic activity which might well be light-inhibited.

Our inability to demonstrate humoral mediation of inhibition or involvement of the testis in inhibition is consistent with the thesis that inhibition is purely a central phenomenon acting on excitatory pathways to the light organ. These negative results can, of course, have no direct bearing on the successful inhibition transfer reported in *Luciola,* although the marked discrepancy of our nor-epinephrine transfer times and the time required to effect inhibition transfer in *Luciola* (over a minute as compared with a few seconds) suggests that factors other than simple diffusion of an inhibitory agent are concerned in the *Luciola* experiments.

Since dim, brief illuminations can bring about effective inhibition (Fig. 2), photic inhibition may play a significant role in flash communication. The flash pattern being of critical importance in communication, photic inhibition might serve to prevent the confusion which might ensue if, for example, two males close together commenced signalling slightly out of phase. Inhibition of the late male by the first flash of the other should prevent signal garbling.

SUMMARY

1. Photic inhibition of spontaneous flashing is demonstrated in *Photuris missouriensis.*

2. The minimum latency of inhibition is between 80 and 160 msec. Inhibition is expressed as completely missed or diminished intensity flashes. There is a tendency for the flashes appearing during inhibition to be later than expected but generally flashing returns to pre-inhibitory timing, suggesting continued operation of a flash pacemaker. The extent of inhibition was found to be light intensity dependent above minimal intensities at which on-off triggering effects are dominant.

3. Direct excitation of the light organ could not be inhibited *via* the visual system.

4. Transfer of inhibition from one *Photuris* to another, *via* saline bridging their body cavities, could not be accomplished.

5. These results, arguing for a purely central mechanism of inhibition in *Photuris,* are compared with those of Magni and Brunelli *et al.,* who propose both central and peripheral inhibition of flashing in *Luciola.*

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FINE STRUCTURES OF THE CARBON MONOXIDE SECRETING TISSUE IN THE FLOAT OF PORTUGUESE MAN-OF-WAR PHYSALIA PHYSALIS L.)[1]

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The Portuguese-man-of-war has been sighted in all seas of the world and has intrigued naturalists for hundreds of years. Much is known about its morphology and general behavior. These aspects have been well summarized in the companion monographs of Totton (1960) and Mackie (1960). Totton carefully outlines the development of the animal which starts as a single hydranth-like form (with one tentacle and a small float) and progresses to a very complex form with modified hydranths budded from but still attached to the original body. He also includes interesting observations on right-hand sailing and left-hand sailing by individuals. His interpretations of the possible significance of the phenomena are at variance with those of Woodcock (1944, 1956).

Despite the long-term interest in *Physalia* very little is known of its physiology. This may be due to the fact that the animal is extremely sensitive to confinement. If placed in an aquarium, it will start degenerating in a day or two. Charles E. Lane, who has made many observations of *Physalia* on the Florida coast off the Institute of Marine Sciences, University of Miami, believes that if the extended tentacles repeatedly touch solid bottom, the animal is adversely affected (personal communication). He feels that a cylindrical sea water tank 50 feet tall, 20 feet wide and with air jets about the periphery of the top to keep the animal centered would probably solve the problem of survival in captivity.

Observations have been made on the gas content of the float by previous workers such as Schloesing and Richard (1896). It remained for Wittenberg (1958, 1960) to first describe significant ratios of carbon dioxide in the gases, ranging up to 8% of the total. This observation has been further explored by Clark and Lane (1961), Wittenberg, Noronha and Silverman (1962), Larimer and Ashby (1962) and Hahn and Copeland (1966).

Another colonial siphonophore, the bathypelagic *Nanomia bijuga* recovered from the deep scattering layers off the California coast, has been reported to possess as much as 90% carbon monoxide in its floats (Pickwell, Barham and Wilton, 1964). This form should receive further attention.

Another instance of carbon monoxide production is in the case of the Pacific bladder kelp (*Nereocystis luetkeana*). This is a kelp that may reach 85 feet in length and have up to 4 liters of gas in the stipe and bulb. Langdon (1917) reports the normal occurrence of an average of 4% (range 1 to 12) carbon monoxide in

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the gas. The gas cavity is sterile and therefore it was concluded that the monoxide gas is produced by the plant in its normal respiration (Rigg and Henry, 1935).

There are many references to carbon monoxide production in other forms but these deal with special circumstances. Metz and Sjöstrand (1954) recorded small amounts of the gas released by guinea pigs and rabbits, probably accounted for by the decomposition of hemoglobin. Wilks (1959) by grinding up alfalfa and putting it in a flask exposed to direct sunlight obtained traces of the gas. Westlake *et al.* (1961) by exposing moulds to various substrates were able to show that the metabolic pathway, under certain circumstances, may use 1 carbon metabolites. Siegel *et al.* (1962) found that seedlings under reduced oxygen tension (5% or less) could produce measurable amounts of carbon monoxide. Loewus and Delwiche (1963) found no carbon monoxide in the floats of the brown alga *Egregia*

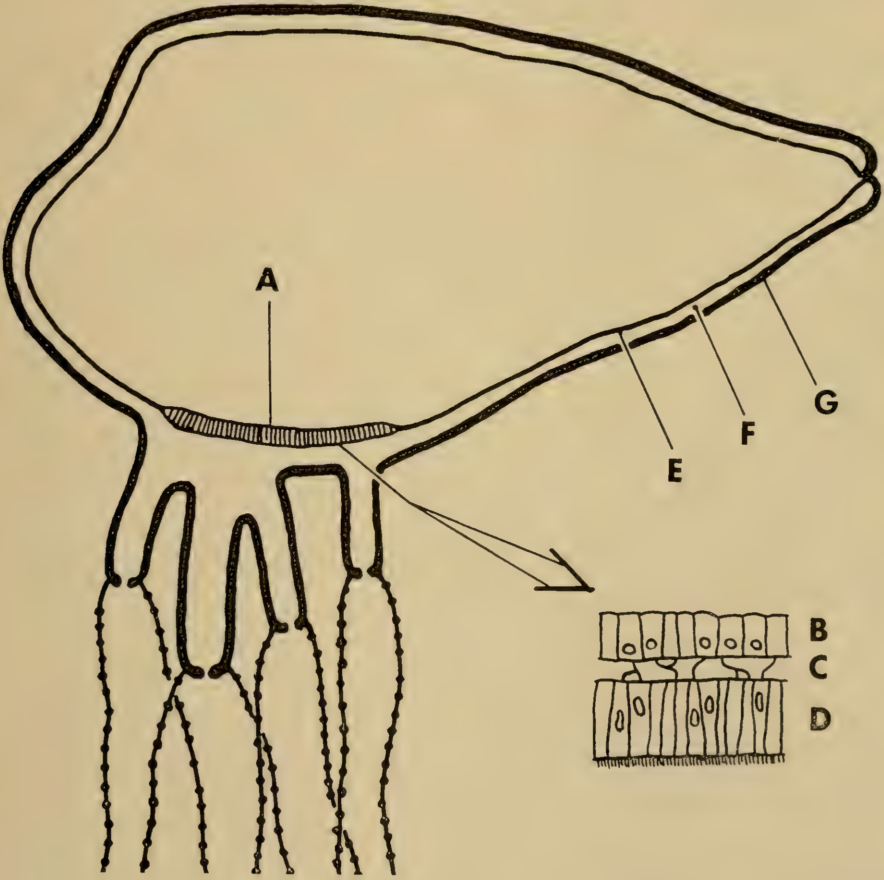


FIGURE 1. Diagram (schematic) of *Physalia* to depict relationship of the gas gland (pneumadena) to the rest of the body. A. The pneumadena (gas gland). B. Ectoderm of pneumadena. C. Mesoglea. D. Gastroderm. E. Pneumatosaccus. F. Gastrovascular cavity. G. Pneumatocodon.

menzies but if they homogenized the alga, carbon monoxide could be produced by the dissociated tissues.

It would therefore appear that the siphonophores *Physalia* and *Nenomia* and the bladder kelp *Nerocystis* are the only forms known to date that produce large amounts of carbon monoxide as part of their normal physiology.

The gas is secreted in *Physalia* by a disc of tissue (pneumadena) on the ventral lining surface of the float (Fig. 1). Again, surprisingly little is known of the histology of the gas-secreting tissue. Incomplete descriptions have been given by Okada (1935) and by Mackie (1960). A description by Dahlgren and Kepner (1908) is probably erroneous.

No description of fine structure observed with the electron microscope has been reported except my own preliminary notes (Copeland, 1962, 1966). This report now presents my morphological observations to date.

MATERIALS AND METHODS

Initial observations were made on *Physalia* collected in the open Atlantic off Gay Head Light, Martha's Vineyard, Massachusetts, in August, 1961. A week of prevailing southerly winds drifted the animals into the area from the Gulf Stream in considerable numbers. Although they appeared normal, it was subsequently discovered that they were in a degenerating condition compared with forms collected in the Gulf of Mexico off the Mississippi River Delta.

Investigations of the physiology of carbon monoxide secretion (Hahn and Copeland, 1966) indicated that the gas-secreting system of *Physalia* is quite sensitive to lowered temperatures and, probably, to physical handling. Therefore, fixation was done aboard boat immediately after collection. The best collecting area was 30 to 50 miles off South Pass, beyond the brown to green Mississippi River fresh water overlay and in the blue, open Gulf water. Gas samples were analyzed for each animal (method, Hahn and Copeland, 1966). Tissues from animals with less than 10% carbon monoxide float gas content were discarded. Concentrations as high as 25–28% were not unusual. The highest recorded concentration was 35% in one individual.

As soon as an animal was netted and the streaming tentacles cut off it was placed on a wire gauze frame in a deep ice chest. The entire float of the animal was thus exposed to ice cold air. After 5 to 10 minutes of cooling, dissection was commenced.

The gas-secreting epithelium is a single cell layer in thickness and easily disrupted by direct injection of fixative into the float and on to the surface of the gland. An osmic fume fixation procedure, as used in the study of gas secretion in the teleost swim bladder (Copeland, 1968), was of some aid but the best fixation procedure involved use of 5% glutaraldehyde (Sabatini *et al.*, 1963) buffered to pH 7.4 with S-collidine buffer (Bennett and Luft, 1959). This was followed by post-fixation in 1% osmic acid in the same buffer. Both fixatives and the intermediate buffer rinse were brought to 950 milliosmoles by the addition of sucrose (Caulfield, 1957).

By use of curved, fine-pointed scissors the pneumatocodon was slit for the length of the animal and the intact inner bladder (pneumatosaccus) was rolled out. The still inflated pneumatosaccus was held with the gas gland (pneumadena)

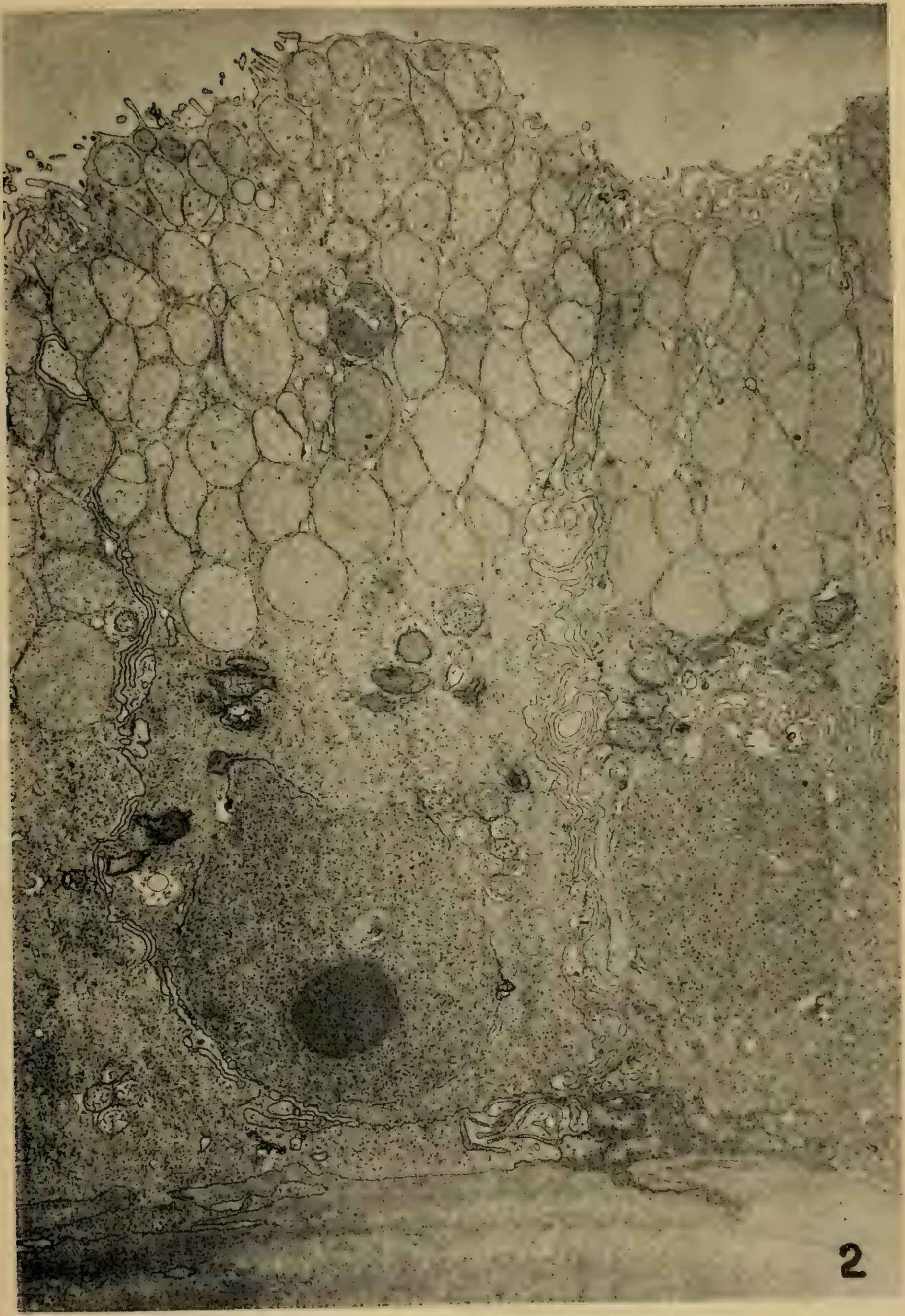
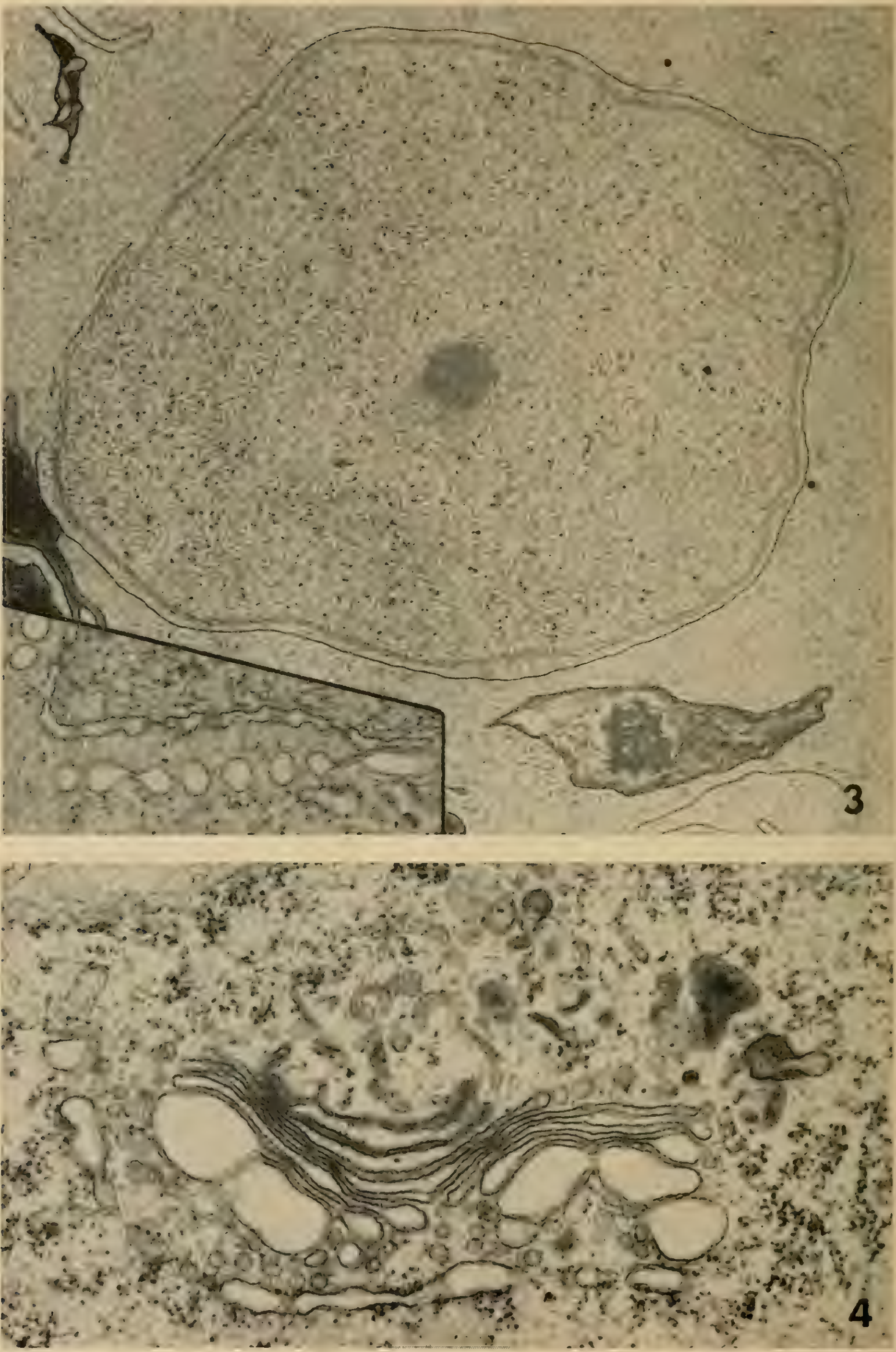


FIGURE 2. Whole cell of pneumadena layer. Gas interface is at top and mesogleal bounding layer is at bottom. See text and other figures for amplification. 4,500 ×.



FIGURES 3–4.

downward and against the surface of the chilled glutaraldehyde fixative. The relatively thick gastroderm and mesoglea were traversed by the fixative before it reached the gas-secreting ectodermal layer. Thus the delicate gas interface surface of the pneumadena was not disturbed by direct contact and the insulating effect of the gastroderm and mesoglea also resulted in more consistent fixation of the ectodermal cells throughout their depth. After 10 minutes of preliminary exposure, some of the same fixative was injected into the cavity of the pneumatosaccus, flooding the ectodermal surface of the pneumadena. After another 10 minutes the pneumatosaccus was lifted from the surface of the fixative, collapsed on dental bite-wax, the pneumadena cut free with a razor blade and placed in a vial to complete a total of 3 hours fixation with glutaraldehyde. Then followed repeated changes of buffer rinse for approximately 2 hours. Post-fixation in 1% osmic acid was for 1 hour. Dehydration was rapid, starting with 50% ethanol and proceeding with constant agitation to absolute in less than an hour. Tissues were then brought to room temperature for final trimming and embedded in Epon 812 by the method of Luft (1961). Thin sections were double stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

RESULTS

The ectodermal layer of the pneumadena is composed of a single layer of columnar cells with basally located nuclei and large, closely packed mitochondria in the distal halves (Fig. 2). The basal surfaces of the cells frequently have extensions that continue into the mesoglea and interdigitate with similar extensions protruding from the ends of the gastroderm cells.

The nuclei are irregular but tend to be spherical or slightly lobate in contour. In the Gay Head Light material the nuclei were frequently surrounded by an even row of vesicles (Copeland, 1962). In the more healthy material of the Gulf of Mexico vesicles were occasionally seen but they were considered to be due to breakdown of a cisternal space frequently seen adjacent and parallel to the nuclear membrane. The cisternal space itself is not always visible but the membranes are quite evident (Fig. 3).

Only scanty endoplasmic reticulum is seen. It is usually of the smooth variety and randomly disposed. A branch of the endoplasmic reticulum is to be observed in association with the Golgi complex when that structure is cut at right angles to its cisternal spaces (Figs. 4 and 5).

The Golgi complexes are usually found just below the mitochondrial zone and have a characteristic configuration (Fig. 4). A flattened unit of the endoplasmic reticulum apparently delivers small vesicles that coalesce into the large cisternal spaces of the Golgi apparatus. The latter then condense and finally release dark, formed bodies at the delivery side of the complex.

A wide range of tinctorial values are to be seen in the cells. Figure 5 illustrates a case of marked contrast. There seems to be no other significant

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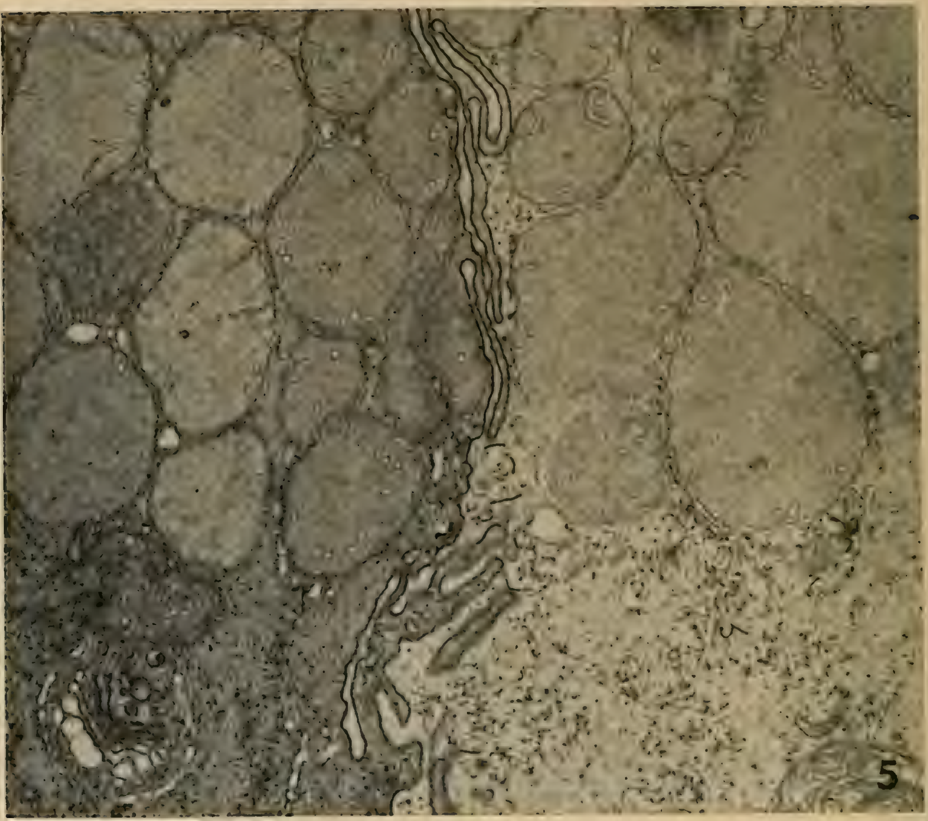
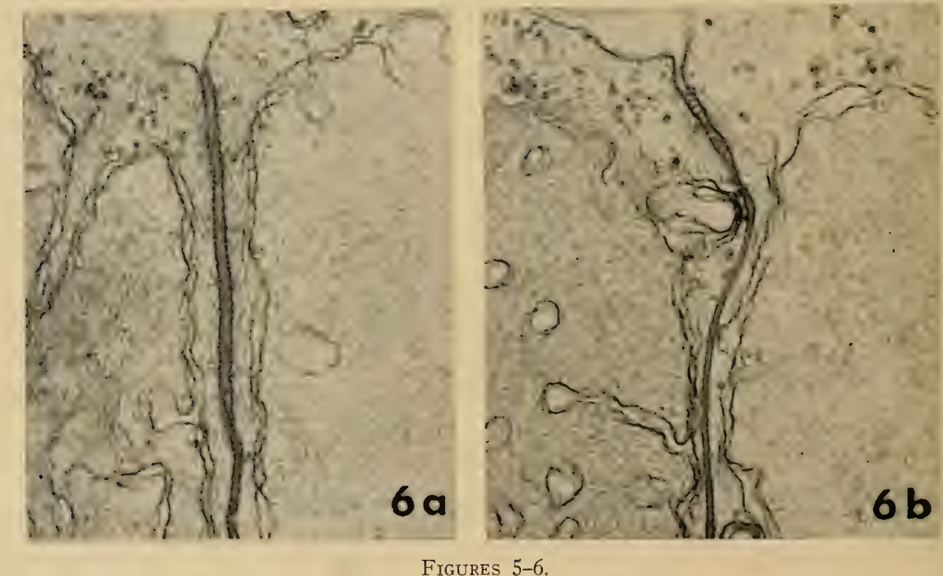
FIGURE 3. Nucleus surrounded by cisternal membranes. Cisternal space has collapsed. Inset shows cisternal space broken into row of vesicles (Gay Head Light, Massachusetts, material). 12,000 ×.

FIGURE 4. Golgi complex. Endoplasmic reticulum space at bottom. Delivery side of complex is toward top. 40,000 ×.





Figures 5–6.

difference between such cells except in the case of extremely dark, obviously moribund cells. Whatever the reason for the tinctorial difference it must be a general one because the entire cell is involved. Figure 5 also well illustrates the mild degree of overlapping interdigitation between the adjoining sides of neighboring cells.

The junction between neighboring cells is plain except for a density between the plasma membranes at the distal ends of the cells near the gas interface (Fig. 6a). Under suitable orientation of sectioning, at least part of the density is seen as a typical septate desmosome (Fig. 6b). Typical zonulae occludentes, zonulae adhaerentes or maculae adhaerentes are not seen.

Immediately below the zone of closely packed mitochondria and sometimes extending down each side of the nucleus is a zone populated by multivesiculate bodies (Fig. 7). These are numerous, usually have multiple encapsulating membranes and internal vesicles that are spherical or oval, sometimes tubular in shape. The multivesicular bodies seem to arise by breakdown of encapsulated mitochondria that become isolated from those above. The initial encapsulation may also trap some of the adjacent cytoplasm (Fig. 7). However, the exact origin and fate of the multivesiculate bodies is not clear at this time.

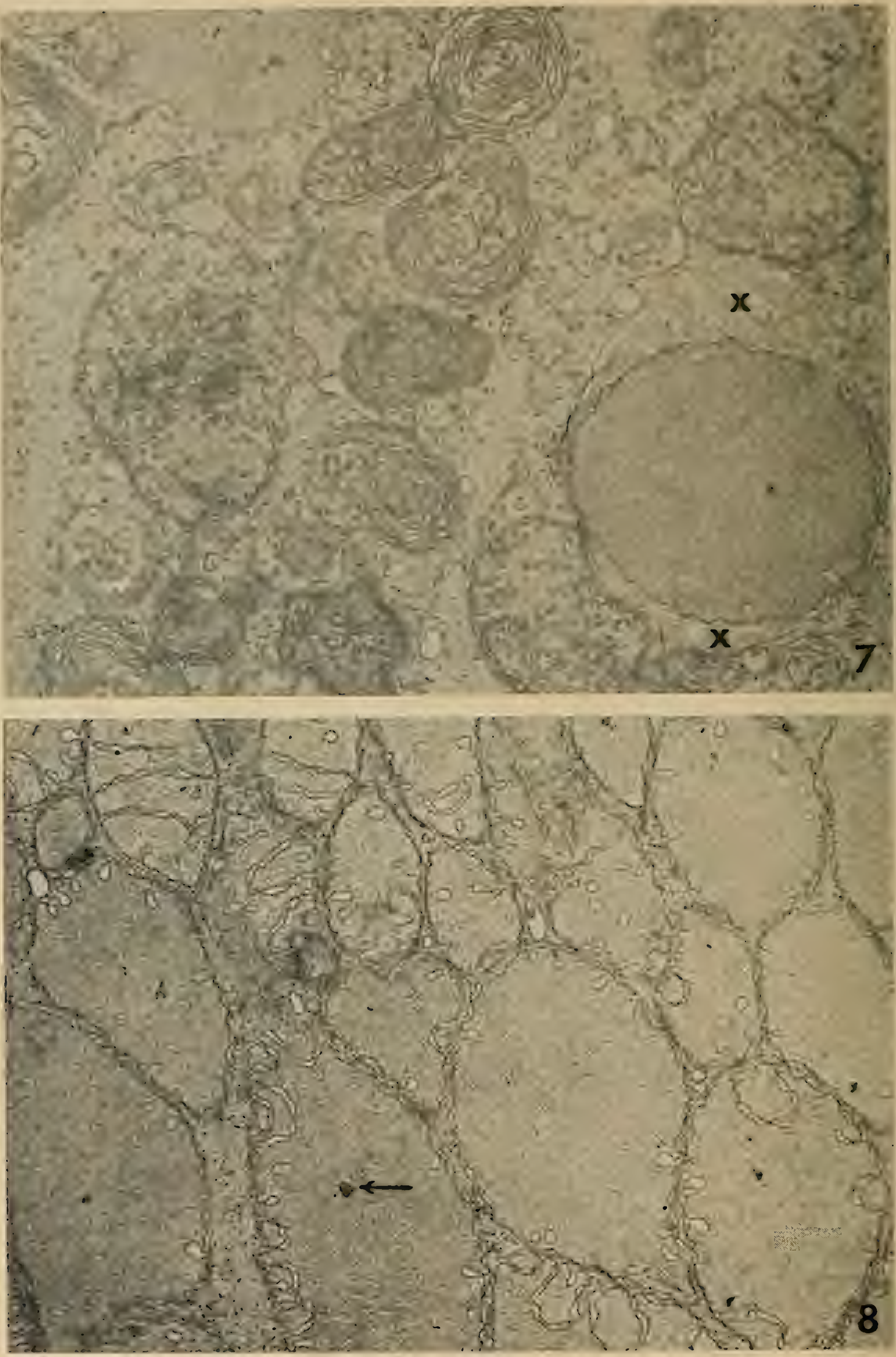
The mitochondria are quite noteworthy. They are oval in form and closely packed in the distal part of the secretory cell (Fig. 8). They differ from most types of mitochondria in having very few cristae. These are short and tubular in the main, but they occasionally form flattened extensions from one side back to the same side or across to the opposite membrane (Fig. 8). Almost the total bulk of the mitochondrion is occupied by a dense granular matrix. Irregular, dark particles are occasionally found in the granular matrix.

In well fixed material the gas surface interface of the secretory cells is seen to have many irregular projections (Fig. 9). These are not regular enough to be called microvilli but there is a resemblance. A few vacuoles or vesicles are seen near the surface but not in sufficient numbers to warrant the certainty that they are occupied with secretion of free gas. Many of them are probably oblique sections of the crypts formed by the bases of the cytoplasmic surface projections.

In a preliminary report (Copeland, 1962) it was stated that the distal end of the secretory cells possessed numerous rows of vesicles possibly devoted to gas release and that the cell was devoid of mitochondria. It was hypothesized that in the presence of high carbon monoxide levels the ectodermal cells had become reliant on respiratory and metabolic support from the gastroderm cells *via* the cytoplasmic bridges across the mesogleal layer. This hypothesis may still have some merit but the interpretation was based on a degenerating condition. The 1962 report was based on material collected in the relatively cold waters off Gay Head Light, Massachusetts. Figure 10 illustrates the morphology characteristic of that material. Figure 11 illustrates an intermediate condition observed in material collected in the Gulf of Mexico which had not degenerated as much.

FIGURE 5. Junction of a light and dark cell. Golgi complex at extreme lower left. Both cells appear normal. Interdigitation clearly shown. 12,000 ×.

FIGURE 6. Cell junctions, seen as parallel plasma membranes with dark substance between them (a). Occasionally the dark substance appears septate (b). Both: 55,000 ×.



Figures 7–8.

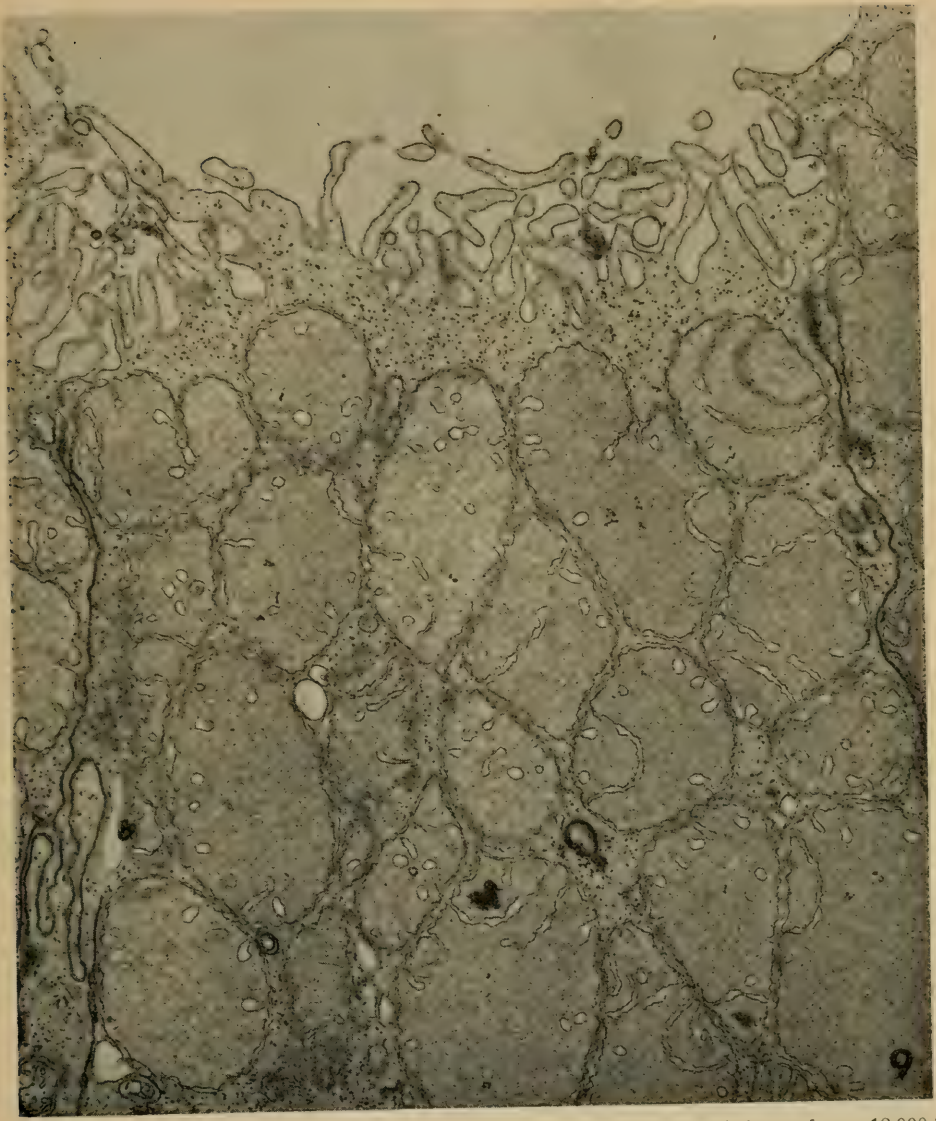
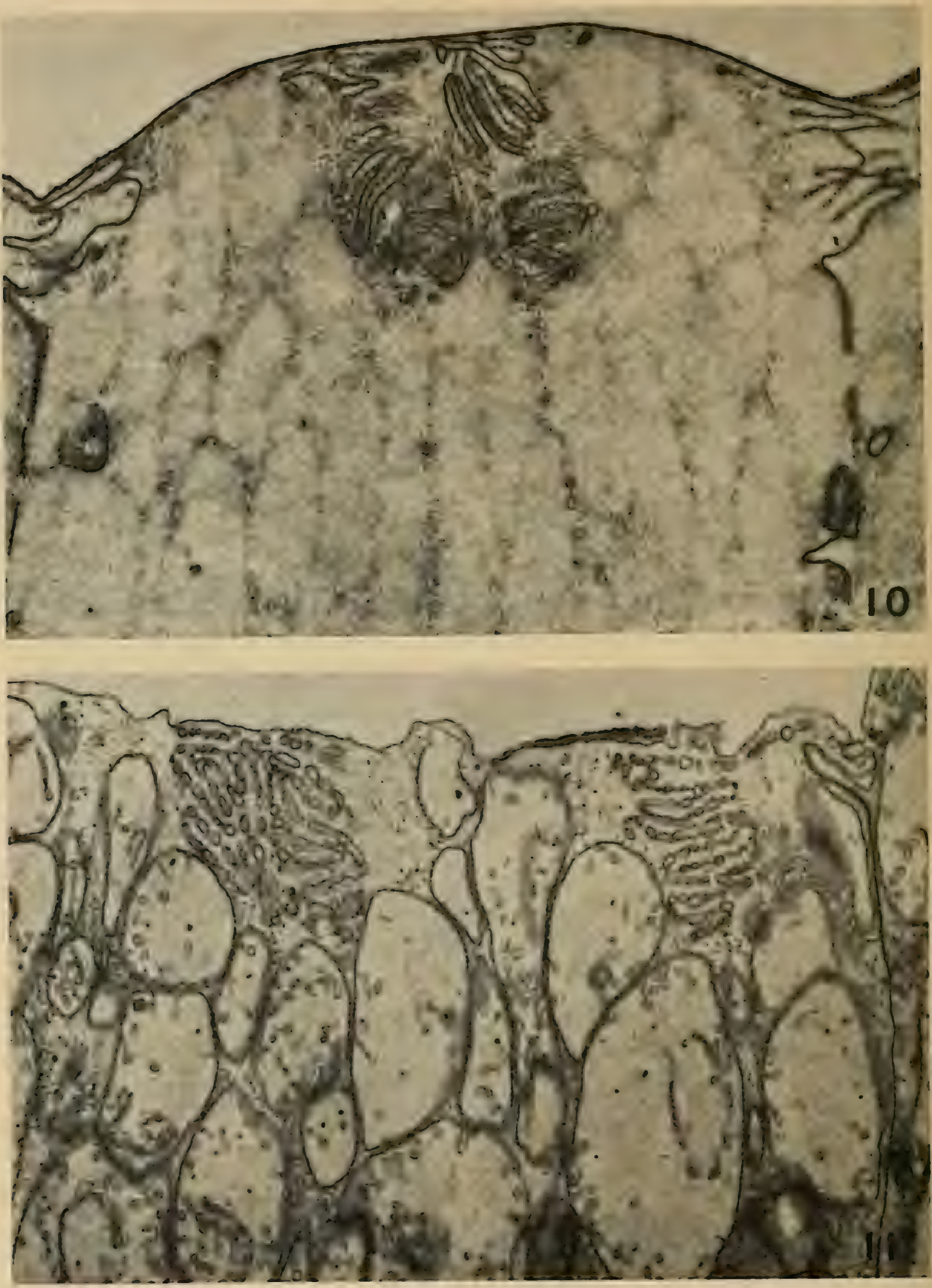


Figure 9. Gas surface interface. Note the many irregular projections of the surface. 18,000 ×

Figure 9 is a comparable view in which no degeneration is visible. It and all the illustrations except Figure 10 are of material collected off the Mississippi River Delta area.

Figure 7. Multivesiculate bodies of varying complexity. Edge of normal mitochondria seen above. To right is an encapsulated mitochondrion with some cytoplasm (X's) included in the capsule. 19,000 ×.

Figure 8. Mitochondria in detail. Cristae quite sparse and short. They are mainly tubular, though long flattened ones are occasionally seen. Occasional, irregular dark bodies are seen in the matrix (arrow). 18,000 ×.



Figures 10–11.

Figure 10. "Degenerate" cell type observed in the material fixed off Gay Head Light, Massachusetts. Mitochondria completely fragmented. "Gas release vesicles" seen just below surface of cell. (Compare with Figures 9 and 11.) 11,500 ×.

As a final observation, it should be stated that the external appearance of the *Physalia* is not a reliable indication of the physiological condition of the pneumadena. The relative percentage of carbon monoxide present in the float gas is, for obvious reasons, a better index.

DISCUSSION

Illustrations of the histological nature of the gas-secreting pneumadena in *Physalia* are scanty indeed. Dahlgren and Kepner (1908) in their Figure 297 illustrated several secreting cells from *Physalia.* However, from my own observations it is evident that they confused the orientation of the ectodermal and gastrodermal layers in their sections and described instead the much longer columnar cells of the gastroderm with its characteristic brush border. This undoubtedly explains why Mackie (1960) was unable to identify the chromatic vacuoles of Dahlgren and Kepner in his sections of the pneumadena prepared by similar methods.

Okada (1935) presented drawings of the early developmental morphology of *Physalia* which depicted the gas gland as having tall columnar cells with basal nuclei and distal brush borders. His drawings were diagrammatic and were not intended to present accurate histological detail.

Mackie (1960) gave a description of the histology of the ectodermal layer of the pneumadena preserved from seven specimens. He described *columnar cells* as being in the majority with occasional *giant cells* scattered among them. In one specimen only he also saw clusters of *islet cells.* Mackie concluded that (p. 391) "The appearance of the cells in the gas gland varies markedly from one specimen to the next, and it is not clear to what extent this variability is due to differing ages of specimens, differing physiological states at the time of fixation or to differing methods of fixation." I have not been able to identify giant cells or islet cells in my own preparations and suspect that Mackie was examining tissues that were in poor condition before fixation.

Carbon monoxide is probably the only gas secreted by the pneumadena (Hahn and Copeland, 1966). Its survival value to the *Physalia* may exist in the fact that the solubility coefficient, and thereby the diffusivity, of the gas is approximately 30 times less than that of the less toxic carbon dioxide and therefore the carbon monoxide gas is more readily retained by the highly hydrated float tissue layers (Hahn and Copeland, 1966). There is no convincing evidence in the present studies that gas is released in the form of bubbles arising within the cytoplasm of the ectodermal cells. Since only one gas may be involved, it can be released readily by direct diffusion from the cell surface. This differs from the situation in the teleost swim bladder where accumulation of multiple molecular species of gases is dependent on physical phenomena associated with cytoplasmic microbubble formation by at least one of the involved gases (Wittenberg, 1958; Copeland, 1968).

The most striking specialization to be seen in the secretory cell is the peculiar

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FIGURE 11. Material fixed in Gulf of Mexico and considered to be transitional between condition in Figures 9 and 10. "Gas release vesicles" similar to those in Figure 10 are seen. Mitochondria, though in poor condition compared with those shown in Figure 9, have not completely degenerated. 11,500 ×.

morphological organization of the mitochondria. The cristae are markedly reduced in size and number. Conversely, the matrix is tremendously hypertrophied in comparison with that in most types of mitochondria. It would be interesting to know if the respiratory enzymes of this peculiar mitochondrion are limited to the membranes. It may also be that the granular matrix in some way is associated with the high concentration of folates observed by Wittenberg, Noronha and Silverman (1962) which are thought to be the probable coenzymes involved in the production of the carbon monoxide gas.

The gas-secreting cell is almost devoid of the cytoplasmic morphology characteristic of most secretory cells. Smooth endoplasmic reticulum characteristic of steroid-producing cells is sparse and the rough variety associated with protein production is particularly elusive. The Golgi complexes are reasonably numerous and are well organized. However, the dark bodies or vesicles from the delivery side of the Golgi complex disappear without formation of anything resembling a secretory granule. The multivesicular bodies are numerous enough to suggest some functional role in the gas secretion. That role could well be one of degradation rather than synthesis, *i.e.,* the bodies may be lysosomal in nature. In short, the gas-secreting cell of the *Physalia* pneumadena is highly specialized for its peculiar function and it is difficult to find cytoplasmic homologies in other cell types.

There is no adequate explanation for the cisternal space, with its bounding membranes, frequently observed parallel to the nuclear membrane. It might in some way serve as a barrier between the nuclear contents and the general cytoplasm for an obscure reason. It may equally well be a rather unusual signal of pending degeneration in those particular cells.

In view of the markedly different observations of the Massachusetts forms as compared with the Mississippi Delta forms, I am reluctant to claim that those from the Delta area are completely normal and healthy animals. Presumably they moved from the Sargassum Sea area of the equatorial Atlantic Ocean on the primary Gulf Stream which swings north through the Yucatan Straits, then eastward to the tip of Florida and then up the Eastern Coast. A branch of this current comes almost straight north from the Yucatan Straits into the Mississippi Delta area *via* the old DeSoto Canyon. In terms of time-distance lapse, the Delta forms should be reasonably healthy, especially the smaller (younger) ones. A quick answer could be obtained by analyzing the gas of *Physalia* collected in their rearing grounds. If concentrations of carbon monoxide higher than 35% are observed, then the fine structure of the pneumadena would require further investigation.

There is one puzzling point not answered by the present study. The animals captured off Gay Head Light, Massachusetts, looked normal externally, with well inflated floats. Unless the permeability of the pneumatosaccus and pneumatocodon had been markedly reduced, the animals were still secreting some gas, albeit at a reduced rate, from tissue in which the mitochondrial membranes had disappeared. If it is assumed that the substrate-enzyme system for carbon monoxide production is associated with the granular matrix of the mitochondria, then that system might still be intact to some degree in the distal ends of the degenerating cells. In that event, the complex of vesicles seen in Figure 10 might bear in last analysis a functional relationship to the modified system to produce a lowered level of gas release.

We are indebted to the Freeport Sulphur Company for the occasional use of their seaplane to locate the blue water interface which is usually in the form of a tide-rip. Its location varies tremendously with wind, tide and the general set of Gulf water movement (which is usually from east to west across the end of the Mississippi River Delta). We are also indebted to the Coast Guard for their most welcome help on two separate emergency occasions.

SUMMARY

1. The carbon monoxide gas-secreting tissue (pneumadena) of *Physalia* is a single layer of ectodermal cells that are cuboidal to columnar in morphology. There is only one specific type cell and it is characterized by having the distal part, adjacent to the gas-interface surface, packed with large mitochondria. The mitochondria are unique in having few and small cristae. Most of the internum of the mitochondrion is occupied by a granular matrix. Compared with other secreting cells, there is very little smooth endoplasmic reticulum and less rough endoplasmic reticulum. There are numerous Golgi complexes and complex multivesiculate bodies. The nucleus is often enveloped by a cisternal space.

2. The secretory epithelium is sensitive to adverse factors such as lowered temperature and presents changes that, though degenerate in appearance, may still have some functional ability to produce carbon monoxide. There is no evidence to indicate conclusively that gas is secreted by formation of vesicles in the cytoplasm. Therefore, it is assumed that carbon monoxide is secreted by diffusion from the cell surface which is frequently thrown into finger-like projections.

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A STUDY OF THE JELLY ENVELOPES SURROUNDING THE EGG OF THE AMPHIBIAN, XENOPUS LAEVIS [1]

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The greatest interest in the oviducal jelly material deposited around amphibian eggs has centered on its possible significance in the processes of meiosis (Humphries, 1961) and fertilization (Hughes, 1957; Kambara, 1953; Katagiri, 1963b, 1966; McLaughlin, 1967; Nadamitsu, 1957; Rugh, 1935, 1951; Shaver and Barch, 1960; Shivers and Metz, 1962; Subtelny and Bradt, 1961; Tchou and Wang, 1956). It is generally accepted that the jelly is necessary for successful fertilization of amphibian eggs; however, the particular properties of the jelly which make its presence essential to fertilization are largely unknown. Kambara (1953, p. 84) has suggested that the jelly " . . . plays the role of a mechanical foothold which enables the sperm to penetrate the eggs." In contrast, Katagiri (1966) proposes that the jelly does not act as a foothold but that a smaller moiety in the jelly may facilitate adherence of the spermatozoa to the egg surface. The recent work of Barbieri and Villeco (1966) also suggests that a small molecular weight substance present in the jelly is necessary for fertilization.

It can be expected that additional knowledge of the structure and composition of the jelly would permit more enlightened studies on precisely what properties of the jelly are necessary for fertilization. Chemical analyses of amphibian jelly have indicated that the main components of the jelly are fucose, hexosamines, one or more hexoses, and protein (Bolognani *et al.*, 1966; Folkes *et al.*, 1950; Hiyama, 1949a, b, c; Kusa and Ozu, 1961; Lee, 1967; Masamune and Yosizawa, 1953; Masamune *et al.*, 1951; Minganti, 1955; Minganti and D'Anna, 1957, 1958; Schulz and Becker, 1935). However, not all of the above investigations dealt with each of these components and in every case all the jelly layers surrounding the egg were analyzed together; no attention was given to the possible differences among layers. That these differences do exist has been shown by histochemical studies on oviducts and eggs (Ghiara, 1960; Humphries, 1966; Humphries and Hughes, 1959; Kambara, 1956a, b, 1957; Kelly, 1954; Salthe, 1963; Shaver, 1966).

The present investigation was undertaken to study the deposition, structure, and composition of the egg jelly of the South African clawed toad, *Xenopus laevis* (Daudin). Since amphibian jelly is deposited in discrete layers, it was considered of prime importance in this study to analyze these layers separately.

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Histochemical methods, which are useful for this purpose, have been employed extensively in the present work. In addition, it appeared that it would be possible with this species to separate the jelly layers in small quantities for chemical analyses. To the author's knowledge, such chemical analyses of individual jelly layers have not been reported for any amphibian.

Materials and Methods

Ovulation of *Xenopus laevis* was induced by an injection of 500 units of chorionic gonadotropin (Antuitrin-S, Parke, Davis).

Cytochemical methods

Oviducts containing eggs were removed from a pithed animal and cut into small segments for fixation. Uterine eggs and deposited eggs were also used. The material was fixed with 10% neutral formalin, 10% neutral formalin containing 0.01 *M* cetylpyridinium chloride (CPC) (Conklin, 1963) or by freeze-substitution (Humphries, 1966). All material was cleared in benzene and embedded in Tissuemat. For most of the staining procedures, serial sections of 10 μ thickness were cut and placed alternately on each of 3 slides.

Carbohydrates. The periodic acid-Schiff reaction was used with and without prior treatment with malt diastase. Both native and boiled enzyme were used in a concentration of 1% in distilled water for 2 hours at room temperature. Toluidine blue was employed in a concentration of 0.1% in distilled water for 1–2 hours. Coriphosphine O (Gurr) was used in a 0.01% solution in distilled water for 15 minutes and sections were examined in distilled water (Humphries, 1966). Detection of fluorescence in coriphosphine-stained material was by means of a Leitz SM microscope using an HBO 200-watt mercury vapor lamp with Leitz exciter filter UV UG1. Alcian blue was employed routinely as a 0.1% solution in 3% acetic acid (pH 2.4–2.6) or was used in increasing concentration of standardized $MgCl_2$ according to the method of Scott and Dorling (1965).

Bovine testicular hyaluronidase (Sigma Chemical Co.) was used in a concentration of 60 USP units/ml. in 0.1 *M* phosphate buffer at pH 6.0. Sections were incubated for 8 hours at 37° C. Cartilage controls were used. Sialidase (neuraminidase, *Vibrio cholerae,* Calbiochem, 500 units/ml.) was used according to the method of Spicer and Warren (1960). Enzyme-treated sections were stained with toluidine blue or alcian blue.

Proteins. Staining was done according to the bromsulfalein method of Silverman and Glick (1966) and the ninhydrin-Schiff procedure of Yasuma and Itchikawa (1953). The dimethylaminobenzaldehyde (DMAB)-nitrite method (Adams, 1957) for tryptophan was also employed. For the detection of sulfhydryl groups the mercury orange method of Bennett and Watts (1958) was used with and without prior treatment with thioglycolate to reduce disulfides (Barka and Anderson, 1963).

Enzymatic treatment of live, jelly-covered eggs

Eggs were stripped from the female and placed immediately into the various solutions. Treatments were carried out at room temperature in native and boiled enzyme solutions and in solvent controls. Papain (2 × crystallized, Sigma Chemical Co.) was used in a concentration of 0.1 mg./ml. in a 0.1 N phosphate buffer (pH 6.3) containing 5 m*M* cysteine-HCL and 5 m*M* EDTA. Pepsin (N. F., Merck and Co.) was employed in a concentration of 1 mg./ml. in 0.01 N HCl. Hyaluronidase was prepared as described above. All observations were made on the live eggs.

Chemical methods

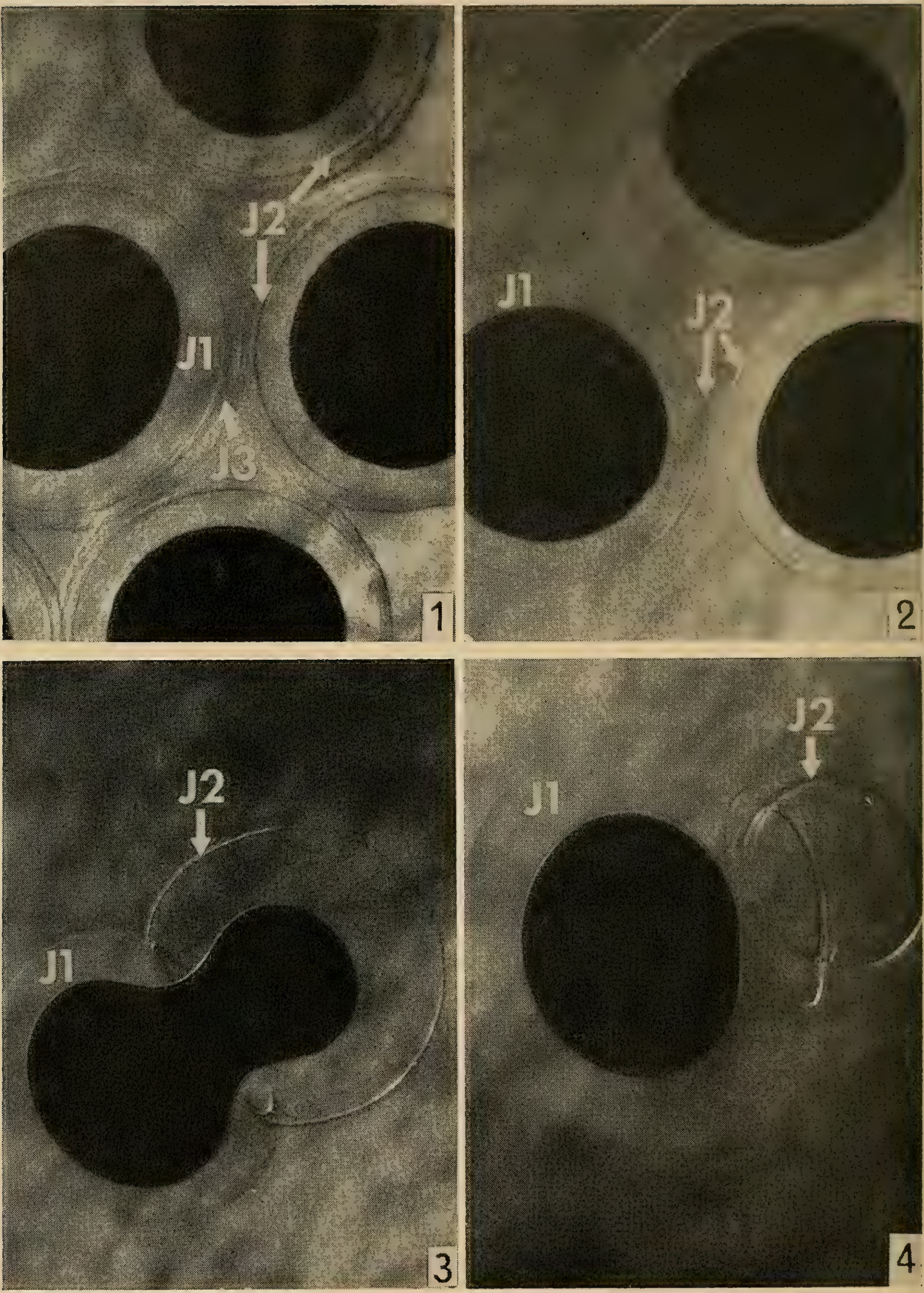
For removal of the jelly, approximately 50 eggs at a time were stripped from a female into distilled water. The jelly layers were removed separately with sharpened watchmaker forceps, placed immediately in vials kept in an ice-salt bath at −10° C. to 0° C. and stored in the frozen state. The jelly coats from the eggs of several females were later pooled and lyophilized.

Nitrogen. Nitrogen content of the jelly was determined by the Nessler method (Lang, 1958) using two different procedures. (1) Equal numbers of coelomic and uterine eggs from the same female were assayed and nitrogen content of the jelly was taken as the difference between the two values. In each case additional eggs from the same female were dried at 100° C. to determine weight. (2) One-mg. samples of lyophilized jelly coats were assayed to determine the distribution of nitrogen among the layers.

Uronic acids. The method of Galambos (1967) was employed. One-mg. samples of each of the separate, lyophilized jelly coats were dissolved in 0.2 ml. of 1 N NaOH, diluted to 2 ml. with distilled water, and 0.8-ml. portions were used for the Galambos procedure. Chondroitin sulfate (20–400 μg., Nutritional Biochemical Corp.) and glucuronic acid (4–80 μg., crystalline, Sigma Chemical Co.) were prepared in 0.1 N NaOH and used as standards.

Sialic acid. Total sialic acid was determined by the thiobarbituric acid assay (Warren, 1959, 1960). To study the distribution of sialic acid among the jelly layers, assays were performed on 1–2 mg. of the separate, lyophilized jelly coats. N-acetyl-neuraminic acid (synthetic, Type IV, Sigma Chemical Co.), in concentrations from 0.01 μmole to 0.05 μmole, was used as the standard. Readings were made on a Zeiss spectrophotometer in 3-ml. cuvettes (1-cm. light path).

Paper chromatography. The method of Caldwell and Pigman (1965) adapted from Masamune and Yosizawa (1953) was used to identify sugars present in the separate jelly layers. Approximately 2 mg. of each coat were hydrolyzed in 1–2 ml. 2 N H_2SO_4 in capped tubes in a boiling water bath for 5 hours. The solutions were then brought to approximately pH 6.0 by adding saturated $BaOH_2$ and, after centrifugation, the supernatants were evaporated to dryness at room temperature with a stream of cool air. The sugars were redissolved in 2–4 drops of distilled water and 8–12 μl. were used to spot each chromatogram. Reference sugars (1 mg./ml.), placed on the same paper, included D-glucose, D-glucosamine-HCL, N-acetyl-D-glucosamine, D-galactose, D-galactosamine-HCl, N-acetyl-D-galactosamine, D-mannose, D-mannosamine-HCl, N-acetyl-D-mannosamine and D-fucose (Sigma Chemical Co.). Descending chromatography was carried out in n-butanol-pyridine-water (5:2:2 v/v) on 18.5 × 22 inch Whatman No. 1 chromatography paper in an equilibrated chromatography chamber. The solvent was allowed to drip off the paper and the total time ranged from 40


J2
J1
J3
1
J1
J2
2
J2
J1
3
J2
J1
4

FIGURES 1–4.

to 62 hours. Reducing sugars were detected by the method of Trevelyan *et al.* (1950) and amino-sugars by an adaptation of the Elson-Morgan reaction for hexosamines (Smith, 1960).

RESULTS

When a uterine egg is placed in tap water the three transparent jelly coats can be observed easily (Fig. 1). The inner layer of jelly (J1) is approximately 0.2 mm. wide and has a gelatinous consistency. The outermost layer (J3) is about 0.1 mm. wide and because it becomes quite sticky in tap water the deposited eggs tend to be held together in masses. Between these two layers is another very thin layer (J2), approximately 0.02 mm. wide, which appears membranous and fairly tough. With forceps, J3 may be removed easily. If J2 is then punctured with the tip of the forceps, the egg, still surrounded by the inner layer (J1), can be pushed out (Figs. 2, 3, 4).

The jelly layers are deposited as the eggs pass through the long, coiled oviduct. As the eggs travel from the coelom into the oviduct they are collected in a thin-walled ostial region. From here they pass singly into the first secreting region where J1 is deposited around the egg. This section of the oviduct is fairly thick-walled and lined with jelly-secreting glands. Approximately half-way down the oviduct the wall becomes even thicker and the glands in this second secreting region can be distinguished histochemically from those in the anterior half of the oviduct. J2 and J3 are formed around the egg as it travels through this region. The eggs then pass into the thin-walled sac-like uterus where they accumulate before egg deposition begins.

Cytochemical observations

The picture observed in the live jelly-covered egg was best retained when the material was fixed by freeze-substitution. Severe shrinkage of the jelly occurred when 10% formalin or 10% formalin containing CPC was used. Since the histochemical reactions were essentially the same after these three fixation methods the material to be described below is that fixed by freeze-substitution unless indicated otherwise.

Both secreting regions of the oviduct and all jelly layers were distinctly PAS-positive. The second secreting region and J2 and J3 reacted more intensely than did the first secreting region and J1. Diastase did not affect the PAS reactivity of the jelly or the oviducal glands but did remove essentially all positivity from the egg. Toluidine blue-stained material in water showed an intense pink to purple metachromasia only in J1 and the glands of the first secreting region. After

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All figures: Live *Xenopus laevis* eggs stripped from a female and immersed in tap water. × 30.

FIGURE 1. All three jelly layers are present.

FIGURE 2. J3 has been removed with forceps.

FIGURE 3. J3 has been removed, J2 has been punctured with forceps, and the egg still surrounded by J1 has been pushed partially out of J2.

FIGURE 4. Same as Figure 3, but egg surrounded by J1 has been pushed completely out of J2. J2 remains on outer edge of J1.

alcohol dehydration of formalin-CPC-fixed material, metachromasia of J1 and the first secreting region was retained although diminished considerably in intensity. In material fixed by freeze-substitution, alcohol dehydration usually abolished any metachromasia. With coriphosphine O, J1 and the glands which secrete it gave a bright orange-red fluorescence. J2, J3, and the glands of the second secreting region were unstained or were only a dull orange. With alcian blue at pH 2.4, J1, and the glands of the first secreting region stained bright blue. The egg, J2, J3, and the second secreting region of the oviduct were unstained.

When material was stained with alcian blue at pH 5.8 in increasing concentrations of $MgCl_2$, J1 and the glands in the first secreting region of the oviduct continued to stain until the $MgCl_2$ concentration exceeded 0.5 *M*–0.7 *M*. Glands of the second secreting region and J2 and J3 did not stain in concentrations above 0.3 *M* $MgCl_2$. Magnesium chloride in excess of 0.2 *M* prevented the egg from staining. Rat rib cartilage staining was abolished in concentrations of $MgCl_2$ above 0.9 *M*.

When any of the carbohydrate stains were used on formalin-CPC-fixed material, the inner portion of J1 gave a more positive reaction than the outer portion of J1. With freeze-substitution J1 appeared essentially homogeneous throughout.

After treatment of sections with hyaluronidase or sialidase, there was no noticeable change in toluidine blue or alcian blue (pH 2.4) staining of the eggs, jelly coats, or oviducal glands.

All four methods employed for the detection of proteins gave the same general staining pattern. With ninhydrin-Schiff the egg was bright magenta. The glands in the first secreting region were essentially unstained, and J1 was only a very pale pink in some sections. J2, J3, and the second secreting region gave a consistent positive reaction although the color was only a pale pink. Somewhat brighter staining was observed after formalin-CPC fixation. Bromsulphalein stained the egg deep purplish-blue. J1 and the first secreting region remained unstained. J2, J3, and the second secreting region were very pale blue. With the DMAB-nitrite method for tryptophan the egg was a positive blue. J1 and the glands which secrete it had only occasional traces of a very pale blue. J3 and the second secreting region were a consistent pale blue. J2 was a deeper blue. When mercury orange was used without prior reduction of disulfides, all three jelly layers and the glands of both secreting regions were negative for sulfhydryl groups. Only the egg stained orange. After thioglycolate treatment, the color in the egg increased and J1 and the first secreting region were pale orange. J2, J3, and the second secreting region were a brighter orange.

Enzymatic treatment of live eggs

After about two hours in papain, J3 had disappeared and J1 had begun to liquefy within the intact J2 layer. After an additional two hours all jelly had been removed. Pepsin appeared to cause dissolution of J3 only. To test further whether J1 and J2 were affected by the enzyme, eggs with only J1 and also separate J2 coats were placed in the pepsin solution. In native enzyme and control solutions J1 shrank but remained around the egg. Separate J2 coats also shrank but, after as long as eight hours, no dissolution of J2 was noticed. Hyaluronidase had no apparent effect on the jelly.

Chemical analyses

When the jelly coats were removed manually from the egg for chemical analyses, it was observed that J3 could be removed without disturbing the other layers and so was probably free of other material. J2 contained a small amount of material from J1 and perhaps also a lesser amount from J3. J1 was thought to be free of contamination from J2 and J3 but, since not all of J1 could be removed without cytolyzing the egg, the innermost part of J1 was not included in the following analyses of the jelly.

Table I gives the values for nitrogen content of: (1) the entire jelly of the eggs of three females (designated A, B, and C); and (2) lyophilized samples of the three separate jelly coats collected from the eggs of several females.

TABLE I

Nitrogen content of eggs and jelly layers

Type of test*	Sample	Mean μg. N/egg (Std. error)	N as % of dry wt. of jelly
(1) Eggs from:			
Female A	coelomic eggs	24.62(±0.40)	6.9%
	uterine eggs	27.05(±0.64)	
Female B	coelomic eggs	46.91(±0.61)	7.9%
	uterine eggs	51.63(±0.22)	
Female C	coelomic eggs	34.63(±0.27)	12.8%
	uterine eggs	38.34(±0.74)	
(2) Lyophilized jelly coats:	J1		7.1%
	J1		7.9%
	J2		8.1%
	J2		6.0%
	J3		6.8%
	J3		7.4%
	J3		6.8%

* See explanation in "Materials and Methods."

Table II gives the results of sialic acid determinations on separate, lyophilized jelly coats. On a dry weight basis J1 appears lowest in sialic acid with 0.14%. J2 has the highest average value, 0.40%, but substantial variations were found in these samples. J3 consistently contained 0.37%. Hydrolysis for three hours did not result in higher sialic acid values but, in fact, lower values were often obtained.

The test for uronic acid content of the three separate jelly layers was negative.

Chromatography

For each jelly layer three separate chromatography runs were made. Duplicate sheets were included in each run so that the two detection reagents could be used.

TABLE II

Sialic acid content of the separate jelly layers

Sample	Number of samples†	Hydrolysis time	Sialic acid μg./mg. dry wt.*	Sialic acid % of dry wt.
J1	3	1 hour	1.39(1.17–1.51)	0.14%
J2	3	1 hour	4.02(2.30–6.00)	0.40%
J3	3	1 hour	3.71	0.37%
J1	1	3 hours	0.62	0.06%
J3	1	3 hours	2.78	0.28%

† Number of samples used to get average values listed.

* Equation 2 of Warren (1959) was used for all calculations and 309, the molecular weight of N-acetylneuraminic acid, was used in order to express concentrations in micrograms.

In Table III are listed the sugars present in each jelly coat as found by comparing the $R_{glucose}$ values of the unknown sugars with those of the standards and by comparing the colors obtained after using the Elson-Morgan reagent.

DISCUSSION

The only three descriptions available for the jelly of *Xenopus laevis* eggs are brief and conflicting. Deuchar (1966) reports two jelly coats, Salthe (1963) suggests five coats, and Ghiara (1960) observed three: a thin inner layer, a wide middle layer, and a thin outer coat. He noted metachromasia in the two inner layers after staining live eggs with toluidine blue. The present report presents evidence that there are three coats: a wide, gelatinous inner coat, a very narrow, membranous middle layer, and a fairly wide outer coat.

The inner layer of jelly appears to contain neutral and acidic polysaccharides and possibly protein. Toluidine blue metachromasia, intense alcian blue staining, and a bright orange-red fluorescence with coriphosphine O strongly suggest the presence of some acid groups whose identity remains unknown. The tests for uronic acids were negative. Similar negative results have been reported for the other amphibians investigated (Bolognani *et al.*, 1966; Folkes *et al.*, 1950; Hiyama, 1949a). It is doubtful that the intense alcian blue (pH 2.4) staining of J1 is due to carboxyl groups because at pH 2.6 or lower, carboxyl group staining may be masked as a result of salt links with proteins (Scott and Dorling, 1965). Using alcian blue at pH 5.8 in increasing concentrations of $MgCL_2$, Scott and Dorling (1965) found that carboxyl group staining can be abolished at a critical electrolyte

TABLE III

Sugars identified in each jelly layer

J1	Fucose, galactose, glucosamine, galactosamine, glucose?*
J2	Fucose, galactose, glucosamine, galactosamine
J3	Fucose, galactose? glucosamine, galactosamine, mannose? mannosamine?

* Question marks in the table indicate that a spot with an R_g value corresponding to that particular known sugar was found in only one of the three chromatography runs.

concentration (CEC) of approximately 0.3 *M* $MgCl_2$ and sulfate at 1.0 *M* or greater; a mixture of the two, such as found in heparin, has an intermediate CEC. The CEC for sialic acid-containing structures is approximately 0.2 *M* $MgCl_2$ (Quintarelli and Dellovo, 1965). Since the CEC of layer J1 is about 0.7 *M*, staining of this layer cannot be accounted for solely by sialic acid. Also, the toluidine blue and the alcian blue stainings were not affected by sialidase treatment. The above evidence and also the persistence of some toluidine blue metachromasia after alcohol dehydration suggest the presence of sulfate in J1 and the glands of the region which secrete it. Past evidence (Minganti, 1955; Lee, 1967) has indicated that amphibian egg jelly has no sulfate. However, other biochemical reports have suggested that sulfate may be present (Minganti and D'Anna, 1958; Bolognani *et al.*, 1966) and, in previous histochemical investigations, several species of amphibians have been found to exhibit metachromasia in the jelly and/or oviducts (Ghiara, 1960; Humphries, 1966; Humphries and Hughes, 1959; Kambara, 1957; Katagiri, 1963a; Kelly, 1954; Shaver, 1966). In no case has the substance responsible for this metachromasia been identified.

The source of the J2 layer is still in question. Although both J2 and J3 appear around the egg in the second secreting region of the oviduct, there has been no way to subdivide further this part of the oviduct and locate precisely the site of deposition of J2. The staining reactions of J2 resemble those of J3 although the reactions for neutral polysaccharides and proteins in J2 are more intense. This may be due in part to the construction of J2, which in sections appears to consist of rather tightly packed fibers arranged parallel to the egg. J2 is not metachromatic and is alcian blue negative at pH 2.4. The alcian blue staining at pH 5.8 is similar to that of J3 and is probably due to amino acids and/or sialic acids, not to uronic acids. Unlike J3, and similar to J1, J2 does not dissolve upon treatment with pepsin. In live material its consistency is quite different from either of the other two layers. It is fairly tough and membranous and not at all sticky as is J3; when J3 is removed, the eggs are no longer held together in masses. Possibly, J2 as such is not deposited by the oviduct but is formed when J3 is deposited on J1. Perhaps some complexing of the components of J1 and J3 occurs, which might change the enzyme susceptibility and staining characteristics of these components. Unless some material is found which is unique to J2 it will be difficult to establish that it is a truly distinct layer and that it is deposited as such by a particular region of the oviduct. There may be a similar situation in the case of *Rana pipiens*, where, although the egg has three jelly layers, the middle part of the oviduct appears immunologically to be an area of overlap between the upper and lower regions (Barch and Shaver, 1963). Katagiri (1963a) describes for the eggs of *Hyla arborea japonica* a "jelly membrane" between the inner and the outer two layers of jelly; as spermatozoa penetrate the jelly they come to a standstill momentarily at this "jelly membrane." Katagiri also suggests that changes in the "jelly membrane" are a prime cause of the decrease in fertilizability of the eggs which have been standing in tap water. Tchou and Wang (1956) noticed that when the eggs of *Bufo bufo asiaticus* remain in water for some time a distinct membrane forms between the outer and inner jelly layers which may act as an obstacle to sperm penetration.

J3 appears to be composed predominantly of neutral polysaccharides and some protein. The presence of carboxyl groups is indicated and, since there is no uronic acid, these groups could be due to amino acids and/or to the small amount of sialic acid that is present. The actual values obtained for sialic acid are probably much too low because fucose, which has been found in all jelly layers, causes abnormally low readings with the method used (Warren, 1959). Recently, sialic acid has also been found in the oviduct and/or egg jelly of other amphibians (Humphries and Workman, 1966; Humphries *et al.*, 1968; Bolognani *et al.*, 1966; Lee, 1967). Soupart and Noyes (1964) demonstrated histochemically the presence of sialic acid in the zona pellucida of the ova of several mammals, and the possible significance of the compound was investigated by Soupart and Clewe (1965) who treated rabbit ova with neuraminidase and found deformation of the zona pellucida and a definite inhibition of sperm penetration.

In view of the differences observed histochemically in the jelly layers of *Xenopus* eggs it is interesting that, qualitatively, the sugar components of the three layers are nearly identical. From the preliminary work reported here, all layers appear to contain fucose, glucosamine, and galactosamine. Galactose is present in J1 and J2 and possibly in J3. In addition, J3 may contain mannose or mannosamine. The sugars may be joined together in polysaccharides and/or may be attached to proteins. It appears from the histochemical reactions that protein is present in all three jelly layers although these tests are not completely satisfactory since they do not actually indicate protein but are specific for certain reactive sites or for particular amino acids. However, proteolytic digestion of the jelly does suggest that the amino acids are linked together in proteins and, furthermore, the enzymatic treatment has served to demonstrate that some differences do exist among the proteins of the three jelly layers.

In conclusion, it is obvious that the jelly capsule is composed of discrete layers. The separate layers appear to differ chemically and physically and as a result they may have quite different functions. To pool all layers for chemical analyses or for studying fertilization may give misleading results. Information about the macromolecules of the jelly layers is now needed.

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SUMMARY

1. The deposition, structure, and composition of the jelly surrounding the egg of *Xenopus laevis* were studied cytochemically and biochemically. During its passage through the oviduct, the egg is invested with three layers of jelly designated J1, J2, and J3, from innermost to outermost. Particular emphasis was placed on analyzing the layers separately.

2. All layers gave positive histochemical tests for neutral polysaccharides. J2 and J3 stained the most intensely. The sugars present in the polysaccharides of each layer were identified chromatographically. J1, J2, and J3 all contain fucose, glucosamine, and galactosamine. Galactose is present in J1 and J2 and possibly in J3. J3 may also contain mannose or mannosamine.

3. On the basis of the histochemical tests it is suggested that J1 contains sulfate. The weak positive reactions for acid polysaccharides in J2 and J3 indicate the presence of carboxyl groups only. Since no uronic acid was found in the jelly it is proposed that the carboxyl groups are part of amino acids and/or sialic acid which was found in small amounts in all layers.

4. The nitrogen values for the three layers are similar. All layers gave positive histochemical tests for protein although the reactions in J1 were weak and irregular. Treatment of live eggs with proteolytic enzymes showed that all jelly layers do contain some protein as an important structural component and that the protein components of the three layers are not identical.

5. It is suggested that future studies should be concerned with characterizing the macromolecules of the jelly and that, for all investigations of the structure and function of the jelly, the differences among layers should be taken into account.

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STUDIES ON THE ENDOCOMMENSAL CILIATE FAUNA OF CARIBBEAN SEA URCHINS

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From a review of the literature, it appears that Jacobs (1914) initiated studies on intestinal ciliates of American sea urchins. During the past 50 years, several investigators have made contributions on the ciliate fauna of sea urchins of the Western Hemisphere; thus almost two dozen species of ciliates have been described from the echinoids. Much of the early work was conducted on sea urchins of Bermuda. Ball (1924) started studies on ciliates of *Diadema* sp. and *Echinometra* sp. of Bermuda and suggested new names for five species of ciliates which she observed; but she never named them. At the request of the Director of the Bermuda Biological Station, Dr. D. H. Wenrich prepared a publication on some of the results of Miss Ball's (now Mrs. Ruth Ball Biggar) study. In the paper of Biggar and Wenrich (1932), the ciliates *Metopus circumlabens* found in *Diadema setosum* and *Echinometra subangularis, Cryptochilum bermudensis* in *Toxopneustes variegatus, Cryptochilum echinometris* of *Echinometris subangularis,* and *Anophrys elongata* found in both *Toxopneustes variegatus* and *Echinometris subangularis* were described. Lucas (1934) described *Metopus rotundus* found in *Diadema setosum* at Bermuda. Since then, several reports have been given on

TABLE I

Frequency of ciliates in echinoids of Puerto Rico

Host	Ciliates							Total number specimens examined
	Anophrys aglycus	*Anophrys elongata*	*Biggaria bermudensis*	*Biggaria echinometris*	*Cohnilembus caeci*	*Cyclidium rhabodtectum*	*Metopus circumladens*	
Diadema antillarum		10−	38++				26++	38
Echinometra lucunter		40++	36++	8−		21+	49++	59
Lytechinus variegatus	12+	42++	35++	10−		9++	34+	46
Tripneustes ventricosus		16−	34++	4−	31+	33++	30+	53

The numbers given in the columns under ciliates in each of the Tables I–V represent the number of hosts found infested with the indicated ciliate; ++ = very abundant, + = many and − = few.

TABLE II

Frequency of ciliates in echinoids of Curacao

Host	Ciliates							Total number specimens examined
	Anophrys aglycus	*Anophrys elongata*	*Biggaria bermudensis*	*Biggaria echinometris*	*Cohnilembus caeci*	*Cyclidium rhabdotectum*	*Metopus circumlabens*	
Diadema antillarum	4+	1+	6+			6+	8+	8
Echinometra lucunter	4+	3+	2++			2+	2+	6
Lytechinus variegatus	1−	1+	1++					1
Tripneustes ventricosus	8+	8++	3+	1−	3+	1−	3+	12

endocommensal ciliates of echinoids inhabiting the waters of the Atlantic, Gulf, and the Pacific Coasts of North America (Powers, 1933, 1935; Berger, 1960; Berger and Profant, 1961; Beers, 1948, 1954, 1961; Lynch, 1929).

In South America, Urdaneta-Morales and Tengler de McLure (1966) studied the ciliates of *Echinometra lucunter, Diadema antillarum, Tripneustes ventricosus* and *Eucidaris tribuloides* of the Federal District of Venezuela. It appears that the report of Berger (1961) on the ciliates of *Diadema antillarum, Clypeaster rosaceus, Echinometra lucunter, Lytechinus variegatus,* and *Tripneustes ventricosus* of Bimini Islands, Bahamas, is the only information available on endocommensal ciliates of sea urchins inhabiting between Bermuda and South America. Because there seemed not to have been any major studies on ciliates of sea urchins between Bimini and Venezuela, a survey was commenced in early January, 1968, to compare the ciliate fauna of sea urchins in this area of the Caribbean with the

TABLE III

Frequency of ciliates in echinoids of St. Croix

Host	Ciliates							Total number specimens examined
	Anophrys aglycus	*Anophrys elongata*	*Biggaria bermudensis*	*Biggaria echinometris*	*Cohnilembus caeci*	*Cyclidium rhabdotectum*	*Metopus circumlabens*	
Diadema antillarum			10++		1−	3+	10++	10
Echinometra lucunter		4+				1+	3++	4
Tripneustes ventricosus		6++	6++		1−	2+	3++	11

TABLE IV

Frequency of ciliates in echinoids of St. Thomas

Host	Ciliates							Total number specimens examined
	Anophrys aglycus	*Anophrys elongata*	*Biggaria bermudensis*	*Biggaria echinometris*	*Cohnilembus caeci*	*Cyclidium rhabdotectum*	*Metopus circumlabens*	
Diadema antillarum		4+	10++		1−	3+	10++	10
Tripneustes ventricosus		6++	6++		1−	2+	3++	11

reports on Bimini and Venezuela. This report concerns the results from studies on the endocommensal ciliates found in four species of sea urchins collected from the Caribbean Sea at St. Thomas, St. Croix of the Virgin Islands, Curacao, Netherlands Antilles, Vieques, and with major emphasis on samples taken from the southwestern coast of Puerto Rico.

MATERIALS AND METHODS

Samples of sea urchins, *Echinometra lucunter, Diadema antillarum, Lytechinus variegatus,* and *Tripneustes ventricosus,* were collected from the littoral in the Caribbean at five islands. Specimens were taken from Brewers Bay at St. Thomas, St. Croix near Buck Island, the Piscardera Bay at Curacao, Vieques, and the southwestern coast of Puerto Rico. In this survey 282 sea urchins were collected at a depth not greater than four feet.

The specimens were examined for intestinal ciliates immediately, or on an average within eight hours after collecting. The method of examination was essentially as described by Lucas (1934). Studies were made primarily on living ciliates, observed both by bright-field and phase contrast microscopy. In a few cases, a dilute solution (1 : 10,000) of Lugol's iodine was employed as a supra-

TABLE V

Frequency of ciliates in echinoids of Vieques

Host	Ciliates							Total number specimens examined
	Anophrys aglycus	*Anophrys elongata*	*Biggaria bermudensis*	*Biggaria echinometris*	*Cohnilembus caeci*	*Cyclidium rhabdotectum*	*Metopus circumlabens*	
Echinometra lucunter	1+	4+		4−		2+	2++	5
Lytechinus variegatus	1+	3+	6++			1+		6
Tripneustes ventricosus			2+				1+	2

TABLE VI

Comparative distribution of ciliates

Host	Ciliates						
	Anophrys aglycus	*Anophrys elongata*	*Biggaria bermudensis*	*Biggaria echinometris*	*Cohnilembus caeci*	*Cyclidium rhabdotectum*	*Metopus circumlabens*
Diadema antillarum	— *V* —	*B* — *P*	*B* *V* *P*	*B* — —	— *V* —	— *V* —	*B* *V* *P*
Echinometra lucunter	— *V* —	*B* *V* *P*	*B* *V* *P*	*B* *V* *P*	— *V* —	— *V* *P*	*B* *V* *P*
Lytechinus variegatus	— *P*	*B* *P*	*B* *P*	*B* *P*	— —	— *P*	— *P*
Tripneustes ventricosus	— *V* —	*B* *V* *P*	*B* *V* *P*	*B* *V* *P*	— *V* *P*	— *V* *P*	— *V* *P*

For each host, indication is given for report of individual ciliates at Bimini (Berger, 1961), Venezuela (Urdaneta-Morales and Tengler de McLure, 1966), and Puerto Rico (the present report).

B = Bimini
V = Venezuela
P = Puerto Rico
— = The species was not reported

vital stain. For more detailed morphological studies some of the ciliates were fixed in Schaudinn's fluid, stained in Heidenhain iron hematoxylin, and mounted in Kleermount xylene solution.

RESULTS

Examinations of intestinal samples from the stated echinoid hosts observed in this study revealed that at least seven ciliates live as endocommensals in the Caribbean. The hosts and ciliates are given in Tables I–V. At St. Croix, *Lytechinus variegatus* was not found in the collecting area, while at St. Thomas, *Lytechinus variegatus* and *Echinometra lucunter* were not found in the area. *Diadema antillarum* was not obtained from the collecting area at Vieques.

DISCUSSION

In Table VI, the results of the present study, Puerto Rico only, have been compared with the reports of Berger (1961), and Urdaneta-Morales and Tengler de McLure (1966) on the intestinal ciliates of sea urchins of Bimini and Venezuela, respectively. Because *Lytechinus variegatus* was not included in the study by Urdaneta-Morales and Tengler de McLure (1966), only the ciliates of Bimini and Puerto Rico can be compared for this sea urchin.

It is of interest to note that *Anophrys aglycus* was reported for *Diadema* *antillarum, Echinometra lucunter* and *Tripneustes ventricosus* of Venezuela but not for the same species at Bimini and Puerto Rico. This ciliate was found in *Lytechinus variegatus* at Puerto Rico, and likewise in *Echinometra lucunter* at Vieques. At Curacao, *Anophrys aglycus* was found in four species of sea urchins. In general, the seven ciliates given in this report are found in two or more species of sea urchins from Bimini, through the Caribbean to South America. There are considerable variations in the ciliate fauna and their abundance at different localities; likewise significant variations in hosts' abundance were observed. Thus it may well be that the diet of the host and the abundance of species living in association may be factors involved in the distribution of endocommensal ciliates.

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SUMMARY

Two hundred and eighty-two sea urchins, *Diadema antillarum, Echinometra lucunter, Lytechinus variegatus,* and *Tripneustes ventricosus,* collected from the littoral in the Caribbean were examined for intestinal ciliates. The specimens were collected from the islands, St. Thomas, St. Croix, Curacao, Vieques, and with major emphasis, the southwestern coast of Puerto Rico. Studies were made primarily on living ciliates with the exception of a few specimens fixed in Schaudinn's fluid and stained with iron hematoxyln; seven ciliates were found: *Anophrys aglycus, Anophrys elongata, Biggaria bermudensis, Biggaria echinometris, Cohnilembus caeci, Cyclidium rhabdotectum,* and *Metopus circumlabens.*

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REGENERATING TISSUES FROM THE COCKROACH, LEUCOPHAEA MADERAE: NERVE REGENERATION IN VITRO[1]

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In an earlier study, Marks and Reinecke (1965) reported the migration of cells from the nerve stump of isolated leg regenerates of the Madeira cockroach, *Leucophaea maderae* (F.), and demonstrated that this growth did not follow the cyclical pattern associated with the molting process that was characteristic of epithelial tissues from the regenerating leg. In the same study, an isolated incident was observed and recorded on film in which cell processes grew out from the proximal end of the 5th mesothoracic nerve of an isolated leg regenerate and made contact with a ganglion that had been placed adjacent to it. Wigglesworth (1965) stated that during post-embryonic development, sensory axons *in vivo* normally differentiated and migrated inward to invade the central ganglion while motor axons emerged from the ganglion itself, and Bodenstein (1957) suggested that the same processes occurred during the healing of a severed nerve. Guthrie (1962, 1967) and Jacklet and Cohen (1967) demonstrated that regeneration of transplanted ganglia and the healing of mesothoracic nerves takes place *in vivo* in the adult cockroach. The recent development of a nutrient medium—M-18 (available from Grand Island Biological Co., Grand Island, N. Y. 14072)—that favors nerve growth and development therefore encouraged us to repeat our earlier *in vitro* experiments to study nerve regeneration in detail. In the present study, we attempted to correlate the regenerative growth processes as observed *in vitro* with normal nerve regeneration as found *in vivo* by other workers.

MATERIALS AND METHODS

The technique for preparing leg regenerates was described in detail by Marks (1968). The mesothoracic legs of late instar nymphs were cut off at the trochantero-femoral articulation 24 hours after molting. Regeneration was allowed to proceed for 8 days, at which time the entire coxa was removed from the insect, and the leg regenerate was dissected under sterile conditions; at the same time, the prothoracic ganglion and gland were removed. The explants, two leg regenerates and a single ganglion, were washed in M-18 nutrient medium, placed in a Rose multipurpose tissue chamber under a strip of dialysis membrane, and the explants so oriented that the stump of the 5th mesothoracic nerve projecting proximally from the leg regenerate was adjacent to the stumps of the lateral nerves

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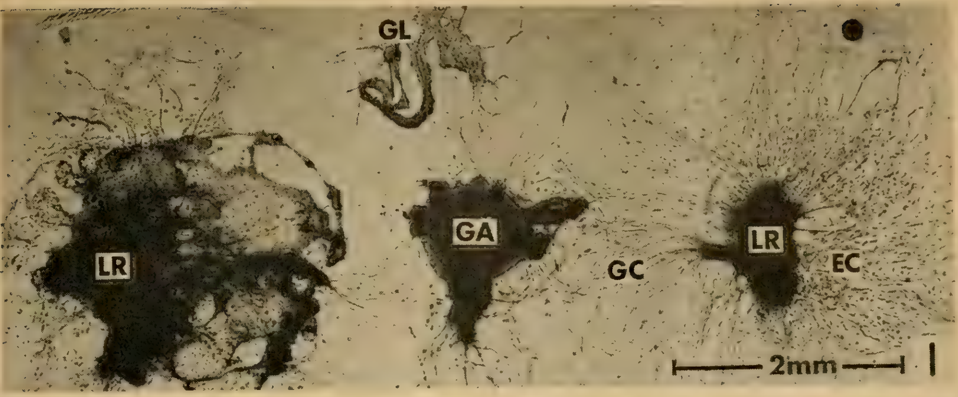
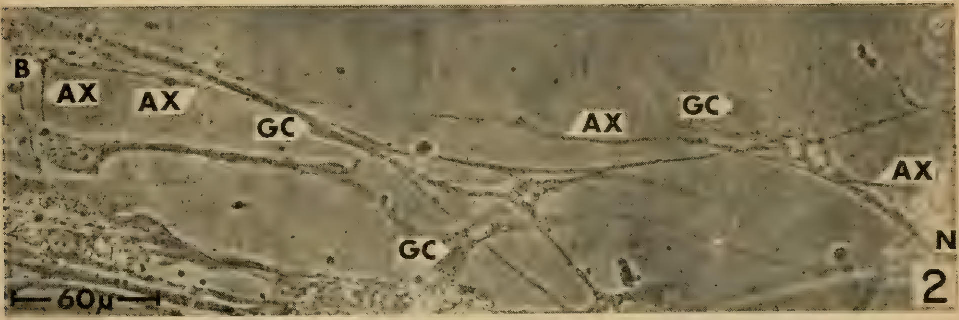
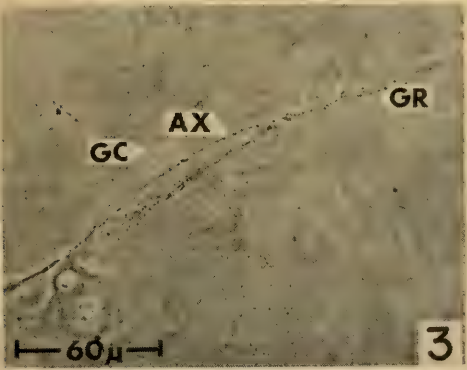
[1] Mention of a proprietary product does not necessarily imply endorsement of this product by the United States Department of Agriculture.







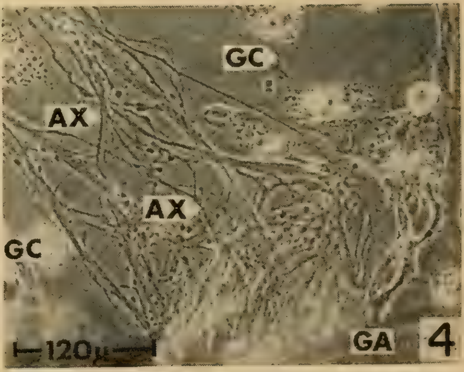


FIGURE 1. Culture containing leg regenerates (lr), prothoracic ganglion (ga), and prothoracic gland (gl). The leg regenerates are surrounded by partial sheets of epithelial cells (ec). Glial cells (gc) are seen emerging from the cut ends of the nerve of both leg regenerates and ganglion and forming a cell bridge. *In vitro* 21 days; transmitted light.

FIGURE 2. Typical growth from the nerve end in a leg regenerate. Sensory axons (ax) emerge from the severed nerve (n) and maintain close contact with several glial cells (gc). *In vitro* 37 days; dark contrast phase.

FIGURE 3. Schwann-like glial cell (gc) with sensory axon (ax) running along the surface. Time-lapse studies show much activity in the region of the growth cone (gr). *In vitro* 60 days; dark contrast phase.

FIGURE 4. Axon (ax) fibers emerging from ganglion explant (ga) migrate through a break in the neurilemma. Glial cells (gc) containing lipid-filled vacuoles are also emerging. *In vitro* 26 days; dark contrast phase.

of the ganglion (Fig. 1). The distance between these tissues was not allowed to exceed 1 mm. In some cases, the prothoracic gland was placed adjacent to the ganglion. The chamber was set up with one glass and one plastic coverslip, and the explants were held against the glass coverslip by the dialysis strip. The completed chamber was filled with M-18 that contained 7.5% fetal calf serum. Another series was prepared with chambers containing only ganglia or leg regenerates. The explants were examined weekly under a phase contrast microscope with a long working distance condenser. If axons and glial cells emerged from the explant, it was scored as positive; if not, it was scored as negative. Only explants that could be clearly scored were included. The results of each experiment were tested against the others by using a test for difference between two sample proportions given by Goldstein (1964). Significance was set at the 95% level of confidence.

To fix and stain these cultures for identification of the cell types, we found it necessary to disassemble the Rose chamber and remove the glass coverslip with its associated dialysis strip. Since the cultures grew between the coverslip and the dialysis strip, extreme care was taken to preserve the delicate structures. First, the 45 × 50-mm. coverslip was cut to a 1-in. width with a diamond pen and then the coverslip was attached, together with the undisturbed dialysis strip, to a slide with metal clips. The entire assembly was then fixed in Gendre's fixative for two hours, stained by the periodic acid-Schiff technique, followed by 5 minutes in Hansen's trioxyhematin. Additional explants were pretreated with either alpha-amylase or pepsin before they were stained by the PAS method. After dehydration and clearing, the dialysis strip was so heavily stained that it was necessary to remove it. In most cases, the explant and associated cell outgrowth adhered to the coverslip though occasionally the explant adhered to the dialysis strip and was pulled away with it. When this occurred, the cell outgrowth remained on the coverslip and could be studied after mounting. The trioxyhematin provided a delicate stain that outlined both the nucleus and cytoplasm, so the location of the PAS-positive granules in the cell was easily determined.

RESULTS

The first activity seen in the explanted leg regenerates occurred within 24 hours when the blood cells migrated onto the coverslip; they frequently formed an imperfect monolayer around the leg regenerate. Within 10 days, epithelial cells were migrating from the explant, and a few days later, large granular cells with long attenuated processes migrated from the nerve sheath or neurilemma and often became very long and spindle-shaped. These probably represent glial cells. They could be distinguished from neurons by their granular cytoplasm and by migration of their cell bodies from the explant across the glass. They often joined in long strings that moved over the coverslip and eventually made contact with cells from other explants, either blood cells or other glial cells (Fig. 1). Examination of the cell surfaces frequently revealed fine fibers of darker contrast. When we traced these fibers, we found that they extended from the interior of the nerve stump. The fibers were morphologically distinct from the glial cells and possessed growth cones identified as sensory on the basis of work done by Pomerat (1967) and Reinecke (personal communication); the apparent location of the cell body in the leg regenerate (*i.e.*, distal to the cut) further identified them as sensory fibers. They sometimes made contact with blood cells as they moved from the explant, but they were usually found closely associated with the glial cells (Fig. 3). In a few cases, they crossed the cell bridge between explants.

The first activity seen in the explanted prothoracic ganglia appeared 12 to 14 days after explantation: blood cells were usually present, but few epithelial cells and no cell sheets were formed around the ganglion. By the 14th day, two types of fibers were migrating from the explant. One emerged from the body of the ganglion through breaks in the neurilemma, and they migrated freely over the glass surface and formed extensive networks. Both blood and glial cells were contacted by these fibers, the growth cones of which were similar to those identified as motor types by Pomerat and Reinecke. The cell bodies of these fibers always remained within the ganglion. Thick ropelike strands often formed and bridged the gap between explants and invaded the tissues of the leg regenerates (Fig. 4).

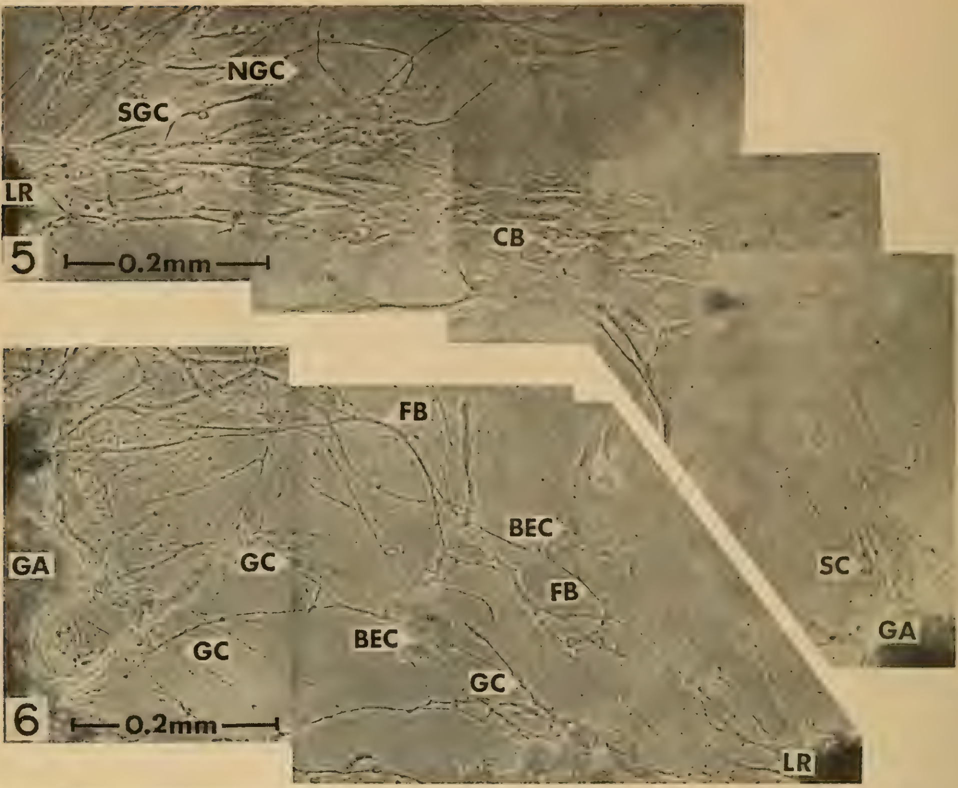
A second type of fiber migrated from the cut ends of the lateral nerves of the ganglion in close association with the glial cells. They were morphologically indistinguishable from the fibers described earlier from the leg regenerate and were assumed to be axons from association neurons within the ganglion. The location of the cell bodies of these fibers is not known.

In the 14 growth chambers containing one ganglion and two leg regenerates, 81% of the 23 leg regenerates that could be scored and 85% of the 14 ganglia showed fiber growth. Cell bridges between the ganglion and one or both the leg regenerates developed in 64% of the 14 chambers (Fig. 5).

Maximum development of the cultures occurred at 25 to 30 days *in vitro;* after this, the supporting cells began to degenerate. The larger groups of nerve fibers persisted longer and remained as a network that could be identified when freed from the accompanying blood and glial cells (Fig. 6). Axoplasmic traffic was clearly visible in time-lapse photomicrographic studies of these fiber bundles. The makeup of the bundles in older chambers was difficult to ascertain in the living material; sensory, motor, or both types of fibers may be involved. A few glial cells usually remained though no well-defined sheath was observed, and there was no evidence of the secretion of a neurilemma. In some of the chambers, such connecting fibers had formed into compact, cable-like structures that resembled a nerve trunk; in others, they remained diffuse. Such nerve-mimicking structures with recognizable axons and glial cells were found as long as 80 days after explantation.

When a prothoracic gland was present with the ganglion, nerve fibers from the ganglion frequently made contact with the gland. The prothoracic gland remained alive and put out cell migrants, and its presence had no visible effect on the activity of either glial cells or nerve fibers.

Of the leg regenerates set up with the ganglion and prothoracic gland, 87% of 16 produced nerve fibers and support cell migrants; of those set up without the gland, 81% of 23 showed such growth. The ganglion explants had a similar pattern: 86% of 13 showed cell migration in the presence of the gland and 85%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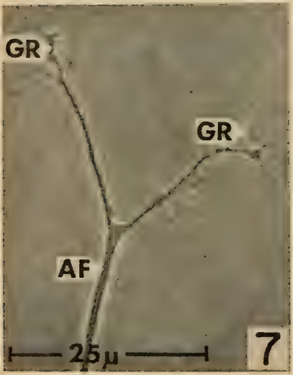
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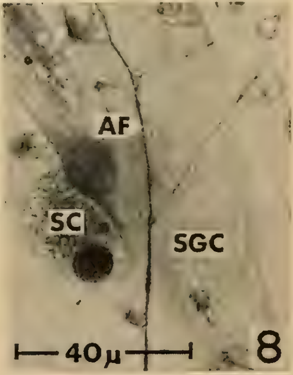
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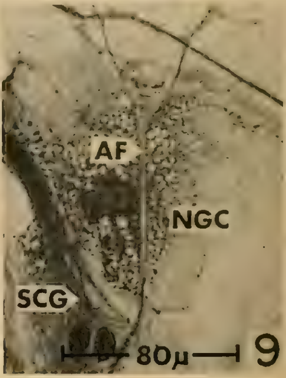
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FIGURE 5. Cell bridge between leg regenerate (lr) and ganglion explant (g) is developing. Elongated Schwann-like glial cells (sgc) and granule-filled nutritive type glial cells (ngc) are emerging from the explants. What may be a sheath cell (sc) is seen near the ganglion explant. The center portion of the bridge (cb) appears to be made up of a mixture of blood, glial, and epithelial cells. *In vitro* 34 days; dark contrast phase.

FIGURE 6. Degeneration of blood and epithelial cells (bec) in old cultures exposes the mature nerve net that has formed. A few recognizable glial cells (gc) remain associated with the fiber bundles (fb). *In vitro* 62 days; dark contrast phase.

of 14 in its absence. A summary of these results is given in Table I. Thus, in this test, the presence of the gland had no apparent effect on the occurrence of nerve regeneration.

To check these observations, we made a second series of experiments in which homologous explants from different animals were placed together in a single chamber. Both leg regenerates (four to a chamber) and prothoracic ganglia (three to a chamber) were prepared as described. The chambers contained about the same amount of tissue as those described earlier. Both kinds of tissue grew quite readily. In chambers containing only leg regenerates, the glial cells and fibers migrated in 44% of the 23 explants. Cell bridges of blood and glial cells that carried axons were formed, but they did not reach and penetrate into other explants, and no lasting fiber aggregates were formed.

TABLE I

The effect of the presence of various tissue combinations on nerve regeneration in vitro

Tissue combinations	Test organs:			
	Leg regenerate		Prothoracic ganglion	
	% growth	Total scored	% growth	Total scored
Leg-ganglion-leg	81	23	85	14
Leg-ganglion+gland-leg	87	16	86	13
4 Leg regenerates	44	23	—	—
3 Ganglia	—	—	90	20

The chambers provided an excellent opportunity to study the regeneration of the sensory type fibers since no motor cell bodies were present in the isolated leg regenerate. The long, slender axons were phase dark and could be seen against the lighter cytoplasm of the glial cells. A single axon sometimes varied throughout its length, broadening and then abruptly narrowing again. Irregular branching occurred, and slender filaments frequently extended laterally at irregular intervals. With time-lapse cinephotomicrography, the growth cone portion of the axon was extremely active, and at times, it extended outward from the advancing glial cell. As the growth cone proceeded along the surface of a sheath cell, it formed flattened droplets of cytoplasm that appeared to provide connections

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FIGURE 7. Branched axon (af) fiber with two growth cones (gr) that are probably sensory in origin but have lost some of their identifying characteristics. This fiber branched from the single large axon bundle that joined a leg regenerate to the ganglion. *In vitro* 74 days; dark contrast phase.

FIGURE 8. Stained preparation showing: a sheath cell (sc), a Schwann-like glial cell (sgc) with cytoplasmic fibers, typical nuclear morphology, and associated axonal fiber (af).

FIGURE 9. Stained preparation showing two Schwann-like glial cells (sgc) adjacent to a nutritive glial cell (ngc) filled with PAS-positive granules. Nuclear morphology of these two cell types is virtually identical. Axonal fibers (af) are closely associated. *In vitro* 50 days.

with the membranes of the sheath cells. Mitochondria and refractile granules were occasionally seen in the axoplasm.

In chambers containing only ganglia, migration of nerve fibers occurred in 90% of the 20 explants, and both types of nerve fibers were present. The results of these experiments are summarized in Table I. In such chambers, particularly those 40 or more days *in vitro*, fibers presumably from association neurons often migrated independently; however, when a glial cell was encountered, the fiber frequently followed it for a considerable distance. In the older chambers, trunk-like aggregates of fibers were frequently formed, possibly as a consequence of the thigmotropic behavior of these cells.

In preparations of young ganglia (15 to 30 days *in vitro*), fibers that were probably motor fibers, as judged by the structure of their growth cones and by the fact that structures of this type were absent in leg regenerate preparations, were found growing mostly from the body of the ganglion through breaks in the neurilemma. They grew out onto the glass surface where the individual fibers formed angular, jointed patterns and loops, and later, as they matured, fiber bundles. In the older chambers (30 to 45 days), it became more difficult to identify the fibers, and growth cones of intermediate types were frequently found (Fig. 7). Cell bridges formed between individual ganglion explants within the same chamber.

While the phase contrast microscope allowed us to ascertain that the cells involved in forming cell bridges were of more than one type, we could not always distinguish clearly between the different types of cells. Several ganglion cultures were therefore fixed and stained for study.

The nerve fibers and glial cells stained readily with Hansen's trioxyhematin, and the neurilemma of the ganglion yielded a positive reaction when it was stained by the PAS technique. The sheath appeared to be lined with small cells containing densely staining nuclei with the chromatin scattered in coarse granules. These cells, often binucleate, occasionally migrated onto the coverslip where they could be identified by their small, dark-staining nuclei (Fig. 8). The cytoplasm was densely packed with PAS-positive granules that were unchanged by digestion with either alpha-amylase or pepsin. The cells occurred in relatively small numbers and fitted Wigglesworth's (1959) description of type I glial cells that belong to the perineurium.

A second type of migrating cell appeared in greater numbers, were much larger, and contained a large, lightly granulated nucleus with one or two prominent nucleoli. The cytoplasm was hyaline around the periphery and frequently included large numbers of granules in the perinuclear region that were also PAS-positive after digestion with alpha-amylase and pepsin (Fig. 9). These cells had an alveolar texture to their cytoplasm, frequently with areas along the periphery that appeared to contain numerous small vacuoles that were also PAS-positive. These cells are one of the components of the cell bridges that form between explants.

A third type of cell present in large numbers varied greatly in shape and was characterized by the presence of numerous fibrils in the cytoplasm and by the absence of PAS-positive granules. They were very large, and some developed long fibrous processes. They were usually the first cells to migrate from a fresh explant, and they were the main components of the cell bridges (Fig. 8). They were also the cells with which the growth cones of the nerve fibers were most commonly associated as they migrated from the explant. Both the second and third types of cells appeared to belong to the type II category of glial cells (Wigglesworth, 1959) and appeared to be actively involved in the process of nerve regeneration. The second type with its PAS-positive granules may play a nutritive role (Wigglesworth, 1960); the third type with its long, fibrous processes and intimate association with the nerve fibers may play the role of Schwann-like cells. It is likely that these two types have a common origin, the morphological differences being dependent on the physiological role of a particular cell at given time since intermediate types are occasionally found.

DISCUSSION

The nerve cell bridges formed in our *in vitro* studies closely resembled those described by Bodenstein (1957). So well, in fact, does his description of *in vivo* regeneration fit the activities found in the present studies that there can be little question that the *in vitro* findings are representative of the process of regeneration as it normally occurs. In both cases, (a) fibers emerge from the proximal and distal nerve stumps and cross a cell bridge made up of blood and connective tissue cells, (b) fiber bundles can be traced from the proximal to the distal nerve stump, (c) many fibers become lost wandering from the target tissues and sometimes form loops and coils that go nowhere, (d) fibers from ganglia and leg regenerate stumps often reunite with other ganglia or leg stumps instead of forming a union between the distal and proximal stumps, and (e) fibers from the distal and proximal stumps may meet in the middle of the cell bridge and form a reticulum of fibers.

Bodenstein also pointed out that nerve regeneration occurred in adult insects and was independent of the molting cycle and thus of the influence of the prothoracic gland. In our study, the prothoracic gland had no visible effect on the growth of nerve fibers from either the leg regenerate or the prothoracic ganglion. This evidence strongly supports the earlier conclusion of Marks and Reinecke (1965) that nerve regeneration is a noncyclical or wound-healing type of growth rather than the cyclical, molting-dependent type found in nymphal epithelial tissues.

One question was whether observable interactions occurred between the leg regenerate and the ganglion tissues. In the cultures containing both types of tissues, fiber growth occurred in 81% of the leg regenerates and 85% of the ganglia. When these same tissues were cultured in isolation, 44% of the regenerates and 90% of the ganglia showed such growth. The results are similar to those from an earlier study (Marks and Reinecke, 1965) in which fiber growth was found in 64% of 11 regenerates when the ganglion was present and in 29% of 24 regenerates when the ganglion was absent. While neither tissue required the presence of the other in order to initiate regenerative growth, the presence of the ganglion significantly increased the occurrence of nerve growth in leg regenerates. However, the presence of the leg regenerate had no measurable effect on the growth from either the ganglion or the nerve stump of other leg regenerates in the chamber. This is evident when these results are compared with those of the 1965 studies. In the earlier study, only two leg regenerates were present in the control chambers, while in the present study, there were four. Yet, in both studies, the nerve growth in the chambers containing only leg regenerates was approximately 50% of that in chambers containing both leg regenerates and ganglia.

Another question concerned the directional orientation of the fibers as they emerged from the explants. During the first few days, the sensory fibers emerging from the distal nerve end in the leg regenerates grew parallel to the shaft of the nerve stump so that they appeared to have a directional orientation if the adjacent explants were close together; however this orientation subsided with time, and additional fibers emerged and grew in numerous directions, apparently independent of any directional influences. Both sensory and motor fibers appeared to have a tactile or thigmotropic response that caused them to follow the cell bridges formed by the blood and sheath cells. If the explants were over a millimeter apart or if no cell bridge formed, most fibers became lost and migrated in a random manner over the glass surface.

One additional phenomenon noted by Bodenstein (1957) was confirmed: those fibers that did eventually make contact with another explant appeared to grow in diameter and persisted in old cultures after the surrounding tissues had broken down. This can be partially explained by the thigmotropic response of the fibers causing bundles to form as new fibers migrated along the surface of the old ones. The migration of fibers from both explants over the same bridge would account for the greater size when compared with bundles formed when fibers migrated from one source only and reached a dead end.

CONCLUSIONS

1. The regeneration of nerve tissues which occurs *in vitro* is comparable, within limits, to that which occurs *in vivo*. Both sensory and motor nerve elements take part in the regenerative process. Blood and glial cells play an important role in forming the cell bridge that facilitates the migration of the nerve axons.

2. The presence of ganglion tissue in the growth chamber exerts a demonstrable stimulating influence on the outgrowth of cells from the nerve stump of the leg regenerate. However, the presence of the leg regenerate appears to have no effect on the outgrowth of cells from the ganglion.

3. A tactile or thigmotropic type of behavior by the migrating axons is evident that explains several of the phenomena described.

4. With such similarity between the development *in vivo* and *in vitro,* the advantages of the *in vitro* technique become apparent. High-resolution photography of the activities of single cells is possible, and the activity can be followed for many days by using time-lapse cinephotomicrography. Also, great experimental flexibility is possible because there need be no interference from other tissues. In addition, tissues from several insects may be included in a single chamber which may make it possible to investigate a number of phenomena that are not otherwise accessible for experimentation.

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RHYTHMIC MOVEMENTS OF CONES IN THE RETINA OF BLUEFISH, POMATOMUS SALTATRIX, HELD IN CONSTANT DARKNESS

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Rods, cones and retinal pigment in a number of fishes undergo alterations of shape and position in response to light and darkness (Arey, 1916; Parker, 1932; Walls, 1942; Nicol, 1965). Several fresh-water and anadromous fish species kept in continuous darkness exhibited well-defined rhythms of photomechanical changes in the retina, implying an internal control mechanism (Welsh and Osborn, 1937; Wigger, 1941; Arey and Mundt, 1941; Ali, 1961; John and Haut, 1964; John, Segall and Zawatzky, 1967; John and Gring, 1968).

We undertook the following study to determine whether the retina of the bluefish *Pomatomus saltatrix* (Linnaeus), a marine pelagic species, undergoes photomechanical changes ordinarily associated with dark- and light-adaption in the absence of light cues. The facts which prompted the study were: (a) under a natural light regime, the bluefish is diurnal and its swimming speed is associated with daily light changes but not entirely controlled by them; (b) rhythms of bluefish swimming activity are evidently in part controlled by internal mechanisms; and (c) the bluefish seems to depend on vision for capturing prey and is particularly active during morning twilight forage (Olla, 1966a, b).

The above facts led us to hypothesize some internal control for adaption in the retina. To investigate this we measured cone and pigment epithelial movement in the retinae of bluefish which we had sampled periodically while the fish were being kept in constant darkness.

MATERIALS AND METHODS

We used bluefish 12 to 17 cm. long captured in Sandy Hook Bay, New Jersey, for this study. We held experimental fish in two 75-gallon recirculating aquaria with water temperature at 22 ± 1° C. and salinity at 25‰. Each aquarium was illuminated by two 24-inch, 20-watt fluorescent lamps. A frosted plate glass was placed between the lamps and water surface to diffuse light evenly. Each aquarium with its lighting system was enclosed in a light-proof box. A phototimer controlled artificial day length.

Before we could establish the presence or absence of a retinal rhythm, it was necessary to determine the range of photomechanical changes in the bluefish retina under both darkness and light and the approximate time necessary to complete these changes. For dark-adaption we subjected five fish to total darkness and removed one fish at intervals of 15, 30, 60, 120, and 180 minutes after the natural seasonal time of sunset (1930 hr.). For light-adaption we subjected five fish to a light intensity of 50 ft.-c. and removed one fish at intervals of 15, 30, 60, 120, and 180 minutes after the natural seasonal time of sunrise (0630 hr.).

Our procedure was as follows: we removed a fish at random from an aquarium, immediately decapitated it, punctured the cornea and fixed the head in Held's solution. This procedure took less than one minute. For fish sampled under darkness, we performed the procedure under illumination from a red-tinted, 15-

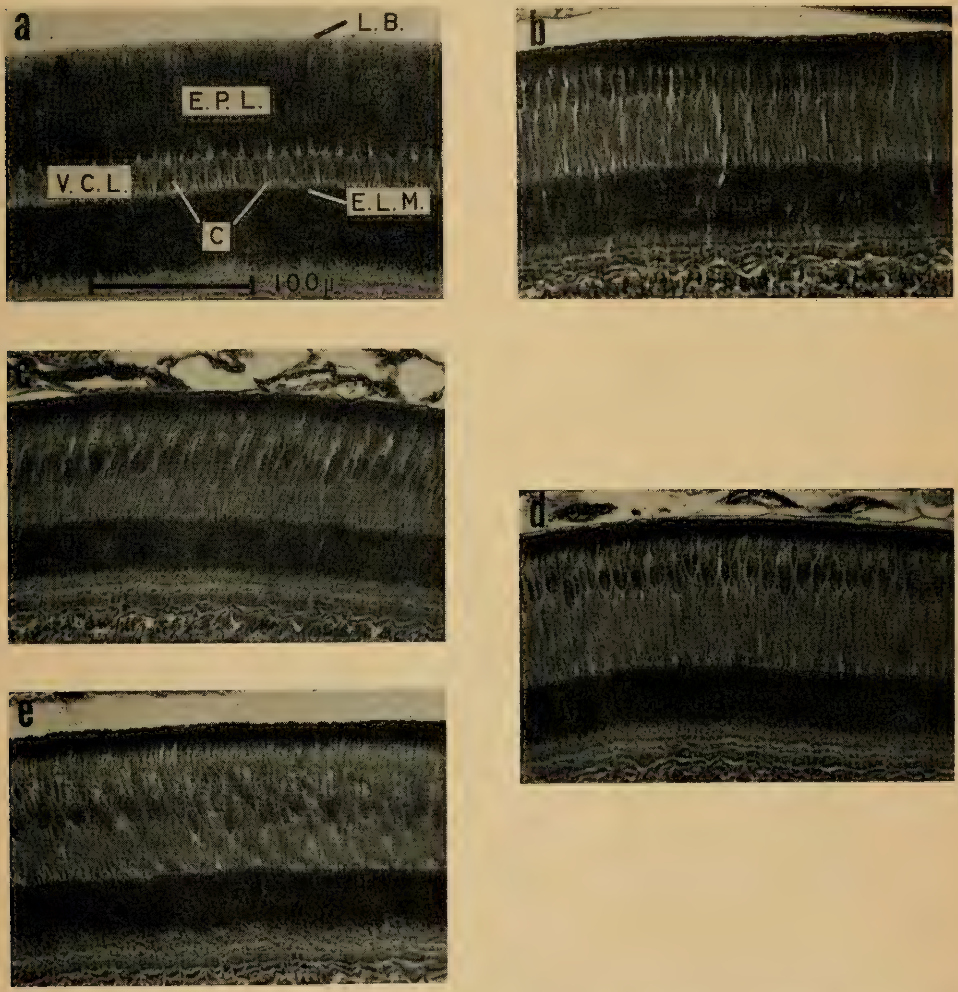

FIGURE 1. Photomicrographs showing the comparison between transverse sections of fully light- and dark-adapted bluefish retinae with those from fish sampled under constant darkness. a. Fully light-adapted retina. b. Fully dark-adapted retina. c. Retina of bluefish sampled at 1200 hours (16$\frac{1}{2}$ hr. after light offset). d. Retina of bluefish sampled at 2400 hours (28$\frac{1}{2}$ hr. after light offset). e. Retina of bluefish sampled at 1200 hours (40$\frac{1}{2}$ hr. after light offset). L.B., lamina basalis; E.P.L., epithelial pigment layer; V.C.L., visual cell layer; E.L.M., external limiting membrane; c, cone ellipsoids.

watt tungsten light bulb. To ensure complete penetration of the fixative, we slit the eyes at the sclerocorneal junction after they had been fixed for 24 hours in darkness. After an additional 24 hours of fixation, we enucleated the heads and removed corneas and lenses. We then dehydrated the eyes in an ethyl alcohol series, cleared them in toluene and embedded them in paraffin (mp 56.5° C.). We sectioned the embedded eyes serially in a transverse plane at 8 μ and stained with Harris' hematoxylin and eosin.

To determine the presence of a retinomotor rhythm under continuous darkness, we held the fish for seven days under an artificial photoperiod of 13 hours light of constant intensity (50 ft.-c.) beginning at 0630 and 11 hours dark beginning at 1930. We assumed that by then the fish had become acclimated to the light cycle. Then beginning at the time the light went off on the seventh day (1930) we held the fish in constant darkness for 49½ hours. At 0900 hours of the eighth day and every three hours thereafter for 36 hours, we sampled two fish from the aquaria and prepared the eyes for histological examination in the same way as described above.

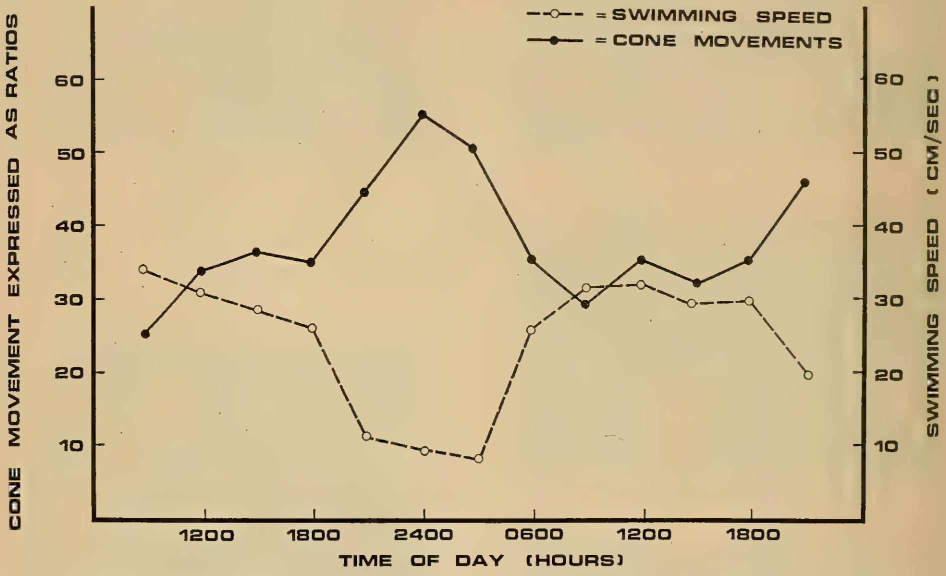

FIGURE 2. Mean values of cone position from fish held in constant darkness compared with swimming activity under constant low illumination (9 ft.-c.).

With a calibrated ocular micrometer we measured the position of 20 consecutive cone ellipsoids from one eye of each fish beginning 50 μ dorsad and 50 μ ventrad to the optic nerve insertion. We expressed measurements as percentages of the width of the combined pigment epithelial and visual cell layers to compensate for variations in retinal cell layer thickness. We did this by dividing the distance from the external limiting membrane to the proximal end of the cone ellipsoid by the distance from the external limiting membrane to the lamina basalis and multiplying the quotient by 100.

RESULTS

We found that the retinal changes associated with light- and dark-adaption followed the classical pattern for the cones and pigment epithelium (Arey, 1916; Parker, 1932; Walls, 1942; Nicol, 1965). Complete light-adaption of the bluefish retina took between 15 and 30 minutes, complete dark-adaption between 30 and 60 minutes. When the fish had become light-adapted, the cone ellipsoids lay close to the external limiting membrane and the pigment epithelium was expanded and remained in that position until the light phase ended (Fig. 1a). When the fish had become dark-adapted the cones lay close to the pigment epithelium which was in a contracted state (Fig. 1b). Thus under a regime of alternating phases of light and dark, the cones and pigment epithelium moved from one extreme to the other with each change of phase. However, under constant darkness the cones continued to move according to the natural photoperiod from the position normally associated with dark-adaption to that normally associated with light-adaption; but they did this gradually in a rhythmic pattern (Fig. 1c–e; Fig. 2). But whereas the pigment epithelium had expanded and

TABLE I

Medians of retinal cone positions expressed as the ratio of the distance between the ELM and the proximal end of the cone ellipsoid/distance between the ELM and the lamina basalis, × 100; and results of the Tukey-Duckworth End Count Test

Time	Fish A		Fish B		Average of medians	End count
	Sampling location		Sampling location			
	Dorsal	Ventral	Dorsal	Ventral		
Clock daytime						
0900	23	24	29	26	26	–
1200	31	31	34	34	33	–
1500	34	37	39	38	37	
1800	39	26	33	34	36	
Clock nighttime						
2100	45	41	43	48	44	+
2400	53	56	56	53	55	+
0300	55	41	50	54	50	+
0600	40	37	32	29	35	
Clock daytime						
0900	26	23	33	34	29	–
1200	35	35	38	30	35	
1500	40	41	25	24	33	–
1800	35	36	38	32	35	
Clock nighttime						
2100	43	46	44	45	45	+

+ = Clock nighttime values larger than the largest clock daytime value.

– = Clock daytime values smaller than the smallest clock nighttime value.

Total end count 8 = 0.025*

* = 0.025 signifies that the differences between the clock daytime and the clock nighttime readings are significant at the 2.5% level.

TABLE II

Sign Test, comparing hourly differences of cone position between fish and between location sampled

Hour	Median: Fish B—Fish A		Median: Ventral sample—Dorsal	
0900	Dorsal, Day 1	+	Fish A, Day 1	+
1200		+		0
1500		+		+
1800		−		−
2100		−		−
2400	Dorsal, Day 2	+	Fish A, Day 2	+
0300		−		−
0600		−		−
0900		+		−
1200		+		0
1500		−		+
1800		+		+
2100		+		+
0900	Ventral, Day 1	+	Fish B, Day 1	−
1200		+		0
1500		+		−
1800		−		+
2100		+		+
2400	Ventral, Day 2	−	Fish B, Day 2	−
0300		+		+
0600		−		−
0900		+		+
1200		−		−
1500		−		−
1800		−		−
2100		−		+
No. of +		14		11
No. of −		12		12

contracted with change from light to darkness, it remained contracted throughout the whole period of constant darkness.

Variation in the positions of the cones relative to the external limiting membrane in any given part of the retina could result from one or both of at least two causes: (a) differences in the part of the retina sampled, and (b) differences between specimens. To examine these points we subjected the data to the following non-parametric statistical analysis: (1) We tabulated the medians of each set of 20 measurements made dorsad and ventrad to the optic nerve insertion for each fish and each sampling hour (Table I). (2) We then tabulated in Table II the signs of the hourly differences in the position of the cones between specimens and between samples taken either dorsad or ventrad to the optic nerve insertion. Applying the Sign Test of Dixon and Mood (1946), we found no significant differences in the position of the cones between fish and sampling location. (3) Therefore, we added the median cone positions for each sampling hour in Table I and computed the averages of the totals. (4) To compare the positions of the cones in specimens sampled during daytime and nighttime, we applied the Tukey-Duckworth End Count Test shown in Table I (Tukey, 1959). The results of this test showed the positions of the cones to differ significantly between the two groups, indicating a movement of cones in specimens kept in total darkness. This could be accounted for only by some degree of internal control. (5) We plotted the mean values of cone positions referred to in (3) above (Fig. 2) to examine the pattern of movement over the period of the experiment and for comparison purposes plotted a curve of the activity rhythm of a group of adult bluefish held under constant low illumination of 9 ft.-c. (Olla, 1966a, b).

It is apparent from examination of the two curves in Figure 2 that the dark-adapted position of the cone ellipsoids and low swimming activity of bluefish occur concurrently during the hours of the natural dark phase and conversely, a light-adapted position of the cone ellipsoids and increased swimming activity occur during the time of the natural light phase. These results provide further evidence for the existence of an internal control mechanism which can act independently of light.

DISCUSSION

Swimming activity and photomechanical changes in the retina are both related to light, are diurnal, and are under some degree of internal control. Previous work on bluefish activity rhythms (Olla, 1966a, b) compared with the present results bears out this relationship (Fig. 2). The fact that an overt response such as swimming speed and a covert response such as cone migration in the retina are in part internally controlled suggests a common synchronizing system for phasing rhythmicity. Internal control of retinal adaption for a predator dependent on vision for prey capture has an obvious selective advantage. It would predispose the retina for light and dark. Such a predisposition would effectively lessen the time for light and dark adaption.

We made no attempt to study the longevity of the retinal rhythm. Previous results of bluefish activity rhythms showed persistence of the rhythm for at least five days. In the absence of daily light cues, an eventual desynchronization of the rhythm might occur. Several investigators have described a reduction in adaption as the length of time under constant darkness increased (Welsh and Osborn, 1937; Wigger, 1941; Ali, 1961; John *et al.*, 1967; John and Gring, 1968).

Although Wigger (1941) in his work on goldfish, *Carassius auratus*, Ali (1961) in Atlantic salmon, *Salmo salar*, and John and Gring (1968) in the bluegill, *Lepomis macrochirus*, found an indication of a rhythm in the pigment epithelium of specimens kept in constant darkness, we found no such rhythm in the bluefish. In some species the pigment epithelium may be controlled internally but evidently in the bluefish it responded only to an external stimulus. A predisposition for light and dark in the epithelial pigment layer may not be as functionally critical as in the visual cells.

Nicol (1965) showed differences in responses between double and single cones in the plaice, *Pleuronectes platessa*, merry sole, *Microstomus kitt*, and sole, *Solea solea*, John *et al.* (1967) in the goldfish, *Carassius auratus*, and John and Gring (1968) in the bluegill, *Lepomis macrochirus*. We observed similar differences between double and single cone movement in the bluefish but did not differentiate between them in our measurements.

Th question of whether retinal rhythmicity in the bluefish becomes changed by manipulation of the light cycle remains for further experiments.

We wish to express our grateful appreciation to Mr. Enoch B. Ferrell for his advice on statistical treatment of the data.

Summary

1. The retina of bluefish, *Pomatomus saltatrix,* undergoes photomechanical changes in response to light and darkness. Complete light-adaption requires between 15 and 30 minutes, complete dark-adaption between 30 and 60 minutes.

2. Retinal cones of juvenile bluefish held under continuous darkness for two days exhibited a diurnal retinomotor rhythm. The epithelial pigment layer remained in a dark-adapted condition throughout the two days and displayed no discernible expansion.

3. Results were related with activity rhythms of bluefish and suggested that internal control of a retinomotor rhythm may predispose the eye to environmental changes in light intensity.

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UREA AND TRIMETHYLAMINE OXIDE LEVELS IN ELASMOBRANCH EMBRYOS

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Urea and trimethylamine oxide (TMAO) have long been recognized as important osmotic constituents of elasmobranch body fluids. Together, these organic solutes make up roughly one-half of the osmotically active constituents of elasmobranchs, allowing them to remain osmotically superior to the surrounding sea water. Eggs of elasmobranchs are nearly isosmotic to sea water (Dakin, 1911), and like the adults, they contain significant quantities of urea (Krukenburg, 1888). Smith (1936), in reviewing urea retention and modes of reproduction in elasmobranchs, concluded that the tendency of elasmobranchs toward intrauterine development may be related to its adaptive advantage in protecting early embryos from urea loss. More recently, Price and Daiber (1967), in their study of the intrauterine environment of ovoviviparous and viviparous forms, have concurred with Smith's conclusion.

In what appears to be the only detailed study of the amounts of urea at various developmental stages of elasmobranchs, Needham and Needham (1930) found that in the dogfish *Scyllium canicula,* low amounts of urea are present when the eggs are laid, and that this urea is added to by the embryos as development proceeds (see also Needham, 1931). Although Needham and Needham did not measure urea concentrations directly, they suggested that urea levels are lower in the undeveloped eggs than in the maternal fluids. They found that only a minimal amount of urea was lost from the developing embryo, and concluded that the increased urea content during development must be due to the excretion of urea by the embryo into the yolk. Their conclusions were rendered uncertain, however, by the lack of a good series of weighings for *Scyllium* embryos, and more importantly by their failure to measure urea in the embryos and yolks separately.

In adult elasmobranchs, the major pathway of urea synthesis appears to be *via* the ornithine-urea cycle (Schooler *et al.,* 1966). Recently (Read, 1968), evidence was presented of an ornithine-urea cycle early in elasmobranch development. Embryos of the oviparous *Raja binoculata* and the ovoviviparous *Squalus suckleyi* were found to contain ornithine carbamoyltransferase and arginase, and the specific activities of these ornithine-urea cycle enzymes underwent marked increases early in development. Neither of these enzymes was found in the yolk, suggesting that any urea formed during development must be supplied by the embryo. This work thus supported the finding of Needham and Needham (1930) that elasmobranch embryos are capable of producing urea.

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In contrast to urea, it is not known whether elasmobranchs are capable of producing TMAO. In what appears to be the only study of TMAO levels in elasmobranch embryos, Goldstein *et al.* (1967) found TMAO in both the yolk and body fluid of embryonic pups, and concluded that active retention of TMAO by the dogfish (*Squalus acanthias*) begins early in development. They were unable to detect synthesis of TMAO by adult elasmobranchs from radioactively labeled compounds (methionine, choline, and trimethylamine), but they did not rule out its synthesis by pathways not involving these compounds. Baker *et al.* (1963) presented evidence supporting a conversion of trimethylamine to TMAO *in vitro* with preparations from elasmobranchs. Cohen *et al.* (1958) found that adult elasmobranchs (*S. acanthias*) starved for up to 41 days were able to retain high blood concentrations of TMAO. Although this finding would indicate that TMAO was being endogenously produced, they suggested that the highly active reabsorption of TMAO by the kidney and its replenishment from TMAO reserves in the muscle could account for its stability.

The present study was undertaken to find out whether either the concentrations or total amounts of urea and TMAO increase during development of the skate *Raja binoculata.* During development, the oviparous elasmobranch embryo appears to be a nutritionally closed system, entirely dependent on its yolk for organic materials. It was felt that if it could be shown that the amount of TMAO is maintained or increases during development, this would indicate that the embryo is capable of producing this compound. In addition, a study was made of urea loss by the different developmental stages of this species.

Materials and Methods

Egg cases of the skate *Raja binoculata* were obtained by otter trawl from the region of Bellingham Bay, Washington. They were transported to the Friday Harbor Laboratories, where they were kept in running, aerated sea water. They were collected on two occasions: late December, 1967, and mid-February, 1968. Egg cases of this species are large, being about 30 cm. in length. Each egg case contains four openings, which during the earliest stages are plugged with solidified albumin. Later the albumin plugs dissolve, allowing sea water to enter. Each egg case contains from one to seven embryos. At any given time during the year a complete range of developmental stages may be obtained, thus facilitating their study.

The measurements made using embryos and their yolks were (i) total volume, (ii) urea concentration and urea content, (iii) TMAO concentration and TMAO content, (iv) dry weight and water content, (v) total urea loss per day, and (vi) freezing point depression of the yolks.

The volumes of the embryos and their yolks were measured separately by their displacement of water. For urea determination a homogenate was prepared using nine times their volume of distilled water. Embryos were homogenized in a Waring Blendor, and yolks were shaken thoroughly with water. In a number of cases, embryos were homogenized together with their yolks, so that the urea concentration in the entire system was measured. The homogenates were then centrifuged for 5 minutes at approximately 600 *g* and a sample of the supernatant taken for urea determination. Urea was measured colorimetrically by nesslerization following treatment with urease. Routine standards were prepared with ammonium sulfate, and the entire method was tested using urea standards.

For TMAO determinations, homogenates were prepared as above using only four volumes of distilled water. The entire homogenate was then extracted with equal its volume of 20% TCA for two hours with constant shaking at room temperature. TMAO was measured by the methods of Dyer (1945) and Dyer *et al.* (1952). Using these methods, the TMAO in the TCA extracts is first reduced using Devarda's alloy to trimethylamine, which is then measured colorimetrically as a picrate salt. It was found necessary to carefully filter the solutions after extraction with TCA and again after reduction with Devarda's alloy to obtain consistent results.

Freezing point depressions of the yolks were measured by the method of Gross (1954). The yolks were first centrifuged at 16,000 *g* for 30 minutes at 4° C. This caused the formation of a small quantity of translucent supernatant, a sample of which could then be used for freezing point determination. Following Krogh (1939), 293 m*M* of NaCl were taken as giving a freezing point depression of 1° C.

Dry weight and water content were determined by placing macerated embryos or yolks in a drying oven at 45° C., and measuring weight loss during consecutive 24-hour periods until no further loss was noted.

In experiments designed to measure urea loss by animals at different developmental stages, embryos were placed separately in covered dishes containing known amounts of Millipore-filtered sea water (usually 250 ml.). Before being placed in the dishes, the embryos were rinsed with additional filtered sea water to reduce the chance of urea loss through the action of diatoms and bacteria. Each dish was well aerated, and after 24 hours urea was measured in samples of sea water taken from the dishes. Urea was measured colorimetrically using 1-phenyl-1,2-propanedione-2-oxime (Archibald, 1945), with urea standards being made up in sea water. Controls included treatment of duplicate samples of the sea water with urease to make certain that urea was indeed being measured. In addition, dishes containing filtered sea water with known quantities of urea were treated exactly as those containing the embryos. There was no significant loss of urea from these dishes during the 24-hour period.

Because of the highly allometric growth pattern of developing skate embryos, length was felt to be an unreliable measure of developmental stage. Values are therefore compared in terms of wet weights of the embryos. The largest of the embryos used in this study still contained yolk in their guts, and were therefore not likely to be taking in nutrients from the environment.

RESULTS

The total amount of urea per embryonic system (embryo plus yolk) was found to increase markedly during development (Fig. 1), confirming a similar finding by Needham and Needham (1930). The concentration of urea in the overall system also increased as a result of a higher urea concentration in the developing embryo than in its yolk (Table I). This overall increase in urea concentration was not, however, great enough to account for the large increase in the total urea content of the system.

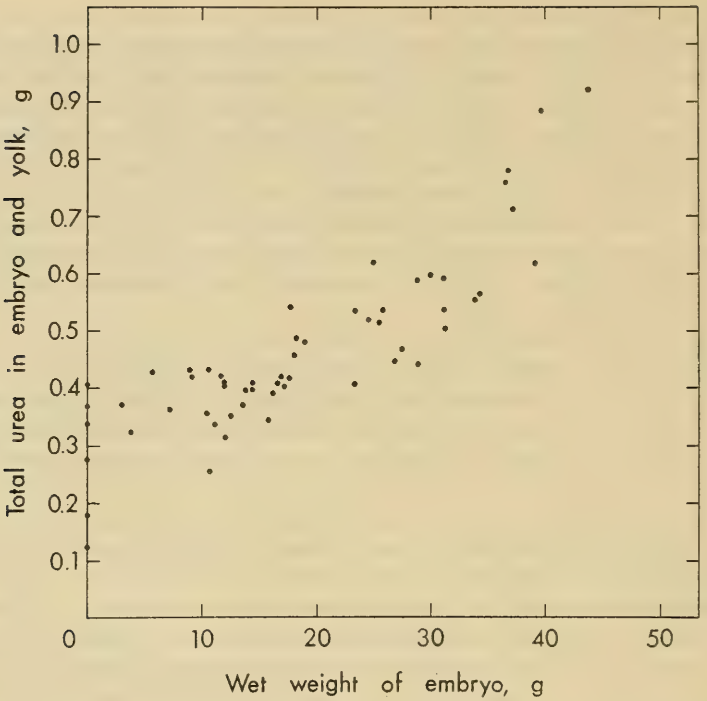

FIGURE 1. Total urea content of the embryonic system (embryo plus yolk) with increasing wet weight (*i.e.*, development) of the embryo. All values in this and remaining figures refer to embryos of the oviparous elasmobranch *Raja binoculata.*

A large increase in the total urea content (Fig. 1) without a similarly large increase in urea concentration can only occur if there is an increase in the total volume of the system during development. This was found to be the case (Fig. 2). As the embryo grows at the expense of its yolk, its volume increases faster than the volume of yolk decreases, leading to a net increase in the total volume. The considerable scatter in the values in Figure 2 reflects variations in the volume of the yolk at any given stage. The final size of the embryos at the time their yolks have been completely absorbed is also highly variable, undoubtedly because of variations in the initial volumes of their yolks.

TABLE I

Concentration of urea and trimethylamine oxide (TMAO) and percentages of water in embryos and yolks of Raja binoculata. *Data are expressed as mean values ± standard deviation, and the number of observations is in parentheses*

	Urea mg.%	TMAO mg.%	Per cent water
Embryos	1817 ± 180 (18)	542.4 ± 119 (28)	77.2 ± 4.6 (10)
Yolks	1242 ± 135 (16)	718.4 ± 107 (28)	51.3 ± 9.1 (15)

The increase in total volume of the system (Fig. 2) was found to be due to an increase in its water content. This, in turn, was correlated with a higher percentage of water in the developing embryo than in its yolk (Table I). The increase in the total volume was gradual, and dependent on the growth of the embryo. The relative percentages of water in embryos and yolks did not change significantly during development. The present finding of an increase in water content confirms the early work of Ranzi (1932), who found that elasmobranch embryos obtain most of the water they require during development from their environment.

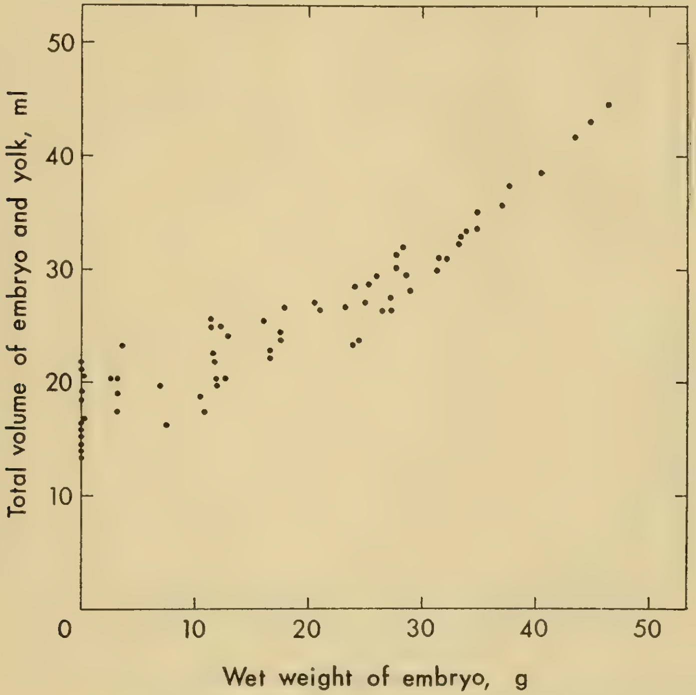

FIGURE 2. Total volume of the embryonic system with increasing wet weight of the embryo.

The difference in urea concentration between embryos and yolks can be correlated with their difference in water content. When the average values for urea concentration in the embryos and yolks are corrected for water content, they are nearly equal (2,354 mg. per 100 ml. water in embryos *versus* 2,420 mg. per 100 ml. water in yolks). Values for urea concentration in the embryos are within the range of urea levels found in various body fluids of adult elasmobranchs (see Bernard *et al.,* 1966). Differences in water content may also help to explain the greater average density of the yolks (1.15 mg./ml.) than of the embryos (1.04 mg./ml.).

The dry weights of embryos plus their yolks ranged between 6.92 and 11.48 g. There did not appear to be a marked change in the dry weight of the system during development, although only ten complete systems were measured. Ranzi (1932) found that embryos of the oviparous elasmobranch, *Scyllium canicula,* show a gain in inorganic substance but a larger loss in organic substance during development. This led to a net loss of approximately 15% in the overall dry weight of the system. At least part of the loss in organic substance observed by Ranzi was probably due to a loss of urea (see below).

The finding that the relative urea concentrations in embryos and yolks remained constant during development is evidence that urea is not excreted into the yolk, as Needham and Needham (1930) suggested. Values for the total loss of urea from the embryonic system into the surrounding sea water are given in Figure 3. The amount of urea lost per day, although highly variable, increased with the increasing size of the embryo. The amount of variation also became greater as development proceeded. The average rate of urea excretion was 0.24 mg./g. wet weight of embryo per day. No urea, however, was detected in sea water or in the albumin surrounding undeveloped eggs. The extremely delicate membrane of these eggs is remarkable in that it appears to be able to retain urea against a tremendous concentration gradient until such time as the embryo is capable of producing urea. It should be emphasized here that the retention of urea by the undeveloped egg appears to lie solely with its delicate membrane. Needham

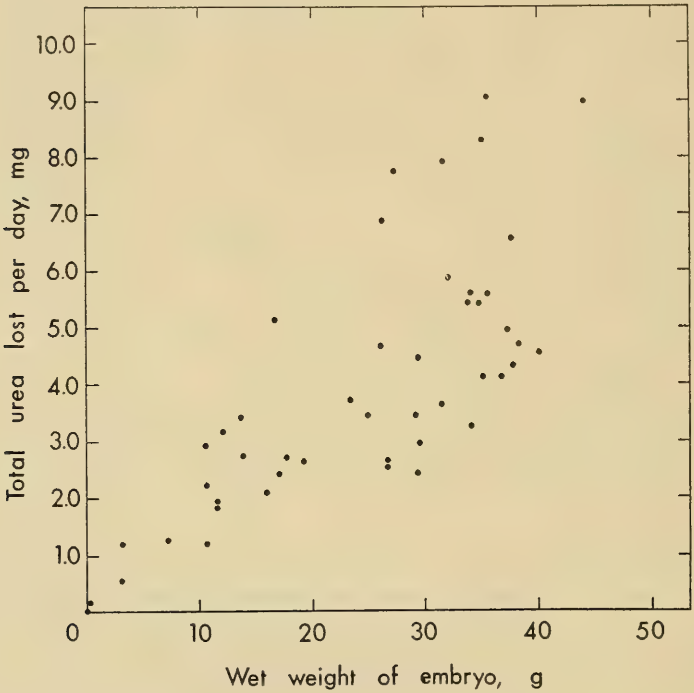

FIGURE 3. Total urea loss per day from the embryonic system with increasing wet weight of the embryo.

and Needham (1930) found that the egg case walls are permeable to urea, so that even in the early stages, when openings in the egg case are plugged with albumin, urea lost by the embryonic system can apparently diffuse to the environment. The enclosure of the eggs in egg cases should therefore not be viewed as a mechanism for urea retention. In the present study it was found that embryos removed from egg cases whose openings were plugged with albumin were able to withstand exposure to sea water for up to a week before losing their integrity and becoming infected with bacteria.

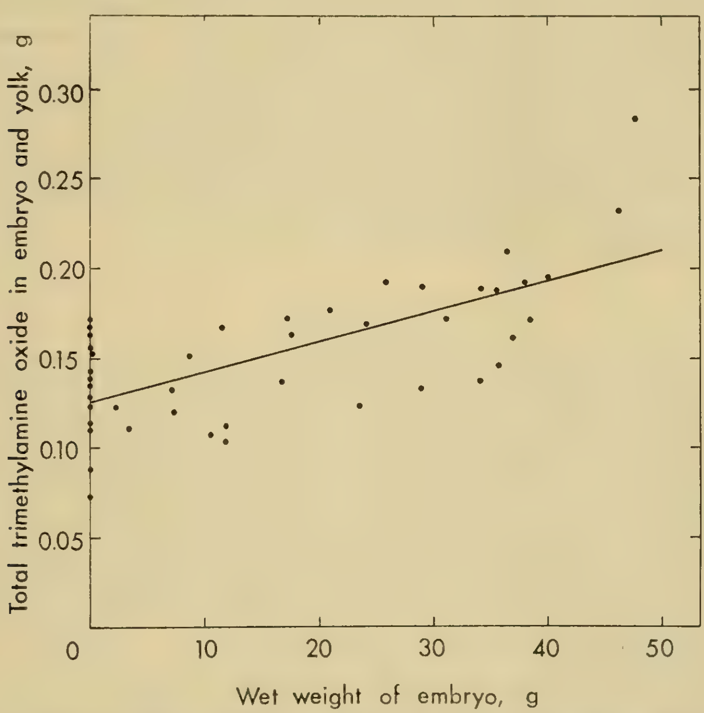

FIGURE 4. Total trimethylamine oxide content of the embryonic system with increasing wet weight of the embryo. The regression line shown has a positive slope of 0.311.

The freezing point depression of the yolks was relatively constant and nearly isosmotic with sea water. The average values for freezing point depression were 1.83 for yolks *versus* 1.82 for sea water.

There was no significant change in the concentrations of TMAO in the embryos and yolks during development (Table I). However, in contrast to urea, TMAO concentrations were found to be consistently higher in yolks than in embryos, even though the difference in the mean values for TMAO (Table I) was not statistically significant. When corrected for water content, the average value for TMAO concentration is twice as high in the yolks as in the embryos (1,410 *versus* 689 mg. per 100 ml. water). The overall concentration of TMAO in the system decreased during development. This decrease was correlated with the lower TMAO concentrations in the developing embryos than in their yolks. This, in turn, meant that the total amount of TMAO did not increase as markedly as did urea. However, there did appear to be a definite increase in total TMAO (Fig. 4). A regression line calculated from the values in Figure 4 had a positive slope of 0.311, which, with a standard error of ± 0.069, differs significantly from zero. (95% confidence interval = 0.202–0.420). The increase in the total volume of the system was greater than the decrease in the TMAO concentration, indicating a net increase in the total amount of TMAO. This increase is evidence that the embryo is capable of producing TMAO, particularly because at these stages the embryo is a closed system with respect to organic substrates. Even if there were no increase in total TMAO, the maintenance of high and relatively stable concentrations of TMAO throughout development would seemingly support the conclusion that TMAO is being produced. Attempts to detect TMAO in the sea water surrounding those embryos used in the experiments designed to measure urea loss were unsuccessful, possibly due to limitations in the sensitivity of the method. The loss of less than about 0.2 mg. of TMAO into the volume of sea water used in these experiments would not have been detected.

Discussion

It seems clear from the present study that embryos of the oviparous elasmobranch *Raja binoculata,* in their ability to retain high urea and TMAO concentrations, have an osmotic relationship to their environment which is similar to that of the adults. The concentration of urea, when measured on the basis of water content, is high and nearly constant throughout development and probably into the adult stage. The concentration of TMAO also remains at a high level, and throughout all stages it is somewhat lower in the embryo than in the yolk. Increases in the total amounts of both urea and TMAO are recognizable mainly through increases in the total volume of the system, since the concentrations of these solutes either decline or remain nearly constant. This increase in volume is in turn due mainly to an increase in water content.

The present report does not in any way invalidate the experimental findings of Needham and Needham (1930), but rather it offers other, and seemingly more reasonable, interpretations of their data. The increase in total urea which they observed during the development of *Scyllium canicula* can be correlated with a correspondingly large increase in water content observed by Ranzi (1932) during development of the same species. Furthermore, Needham and Needham were able to detect urea in the sea water surrounding embryos studied in urea-loss experiments, but they concluded that the amount of urea lost was not significant when compared to the large quantities contained within the embryos. From an osmotic viewpoint, continual loss of even a small amount of urea may be highly significant, particularly if measured over the entire developmental period.

The large variation in the amount of urea lost by embryos of any given developmental stage (Fig. 3), and the relatively stable internal urea concentrations (Table I) may indicate that the production of urea and the utilization of nitrogen-containing compounds are themselves variable, and that the level of urea is controlled at the site or sites of urea loss rather than at the sites of production.[2] It seems apparent that urea produced during the development of elasmobranch embryos serves as both an osmoregulatory and an end product of metabolism. It should be noted, however, that the conditions under which the urea-loss experiments were run may differ markedly from conditions within the egg case, particularly with regard to the amount of oxygen and carbon dioxide as well as conditions of light and trauma. Possible sites of urea loss were not sought in the present study. Perhaps the most obvious site would be the gill filaments, which are highly developed at an early stage.

The finding of high concentrations of TMAO in even the earliest developmental stages indicates that TMAO, like urea, is retained throughout the elasmobranch life-cycle. The finding that the concentration of TMAO in the yolk is higher than in the embryo, particularly when based on water content, may indicate that this compound is taking the place of osmotic constituents which are at lower levels in the yolk than in the body fluids. However, it is possible that some of the TMAO is bound in the yolk and therefore not osmotically active. The significant increase in total TMAO found in these embryos would appear to be the strongest evidence put forth to date that any elasmobranch is capable of producing this compound.

In reviewing the role of urea in elasmobranchs, Smith (1936) concluded that the tendency of elasmobranchs toward intrauterine development (ovoviviparous and viviparous forms) might be explained by the advantage conferred by this pattern of development in allowing the embryos to better retain concentrations of urea. This view has more recently been supported by Price and Daiber (1967) who concluded (p. 259) that "the inability of the embryo to regulate urea and osmotic pressure in early development has been a selection pressure leading the elasmobranchs toward viviparous reproduction." As seen in the present study, both the embryo and the undeveloped egg are apparently able to retain urea against the diffusion gradient which exists between them and either the albumin or sea water. Furthermore, levels of both TMAO and urea are maintained within relatively narrow limits, which suggests strongly that regulation is occurring throughout development. It seems more likely, therefore, that the initial selective pressure toward intrauterine development resulted from factors other than the need to retain urea (*e.g.,* protection against predation and mechanical injury). Once having adopted an ovoviviparous mode of development there may then have been other selective pressures to maintain closer nutritional ties with the maternal organism.

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[2] Studies which I have recently completed indicate that, at least in adult elasmobranchs, there may be a number of possible sites of urea production in addition to the liver. Activities of both ornithine carbamoyltransferase and arginase are clearly detectable in a number of tissues, particularly the spleen and kidney.

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SUMMARY

1. The total amounts of urea and trimethylamine oxide (TMAO) increase during the development of the oviparous elasmobranch *Raja binoculata.* These increases are not explained by increases in the concentrations, which, when measured on the basis of water content, either decline (TMAO) or remain relatively constant (urea).

2. The increases in urea and TMAO are correlated with an increase in the total volume of the embryonic system during development.

3. The increase in the total volume of the system is due mainly to an increase in its water content. This, in turn, is correlated with a higher percentage of water in the developing embryo than in its yolk.

4. The increase in the total amount of TMAO during development would strongly suggest that the embryo is capable of producing this compound, particularly since the embryo is apparently a closed system with regard to organic subtrates.

5. There was a definite, though variable, loss of urea from the embryonic system but not from the undeveloped egg. There was, however, no detectable loss of TMAO. The amount of urea lost per day and the degree of variability increased during development. The undeveloped egg is apparently able to retain urea until such time as the embryo is capable of producing this compound.

6. The maintenance of relatively constant urea and TMAO concentrations implies that regulation occurs throughout development, and that these organic solutes play a similar osmotic role in the embryonic system as in the adult.

7. The ability to retain relatively constant urea levels by even the earliest stages of this oviparous form would seem to make less tenable the suggestion of other authors that the need to retain urea was a selective pressure leading the elasmobranchs toward intrauterine development.

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PEDAL EXPANSION IN THE NATICID SNAILS.
I. INTRODUCTION AND WEIGHING EXPERIMENTS [1]

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Possession of an enormously expanded foot, partially enclosing the shell, is characteristic of living naticid snails (or "moon-snails"), and is commonly employed as a diagnostic feature for the group. The adaptive significance of this apparently disproportionate pedal expansion is two-fold. First, the expanded wedge-shaped propodium and the metapodial folds covering the shell obviously contribute to the efficiency of "plowing" locomotion, as the moon-snail moves along on, and through, sandy substrata. Secondly, the naticids are predaceous carnivores with protrusible proboscides, and employ their extensible pedal lobes to manipulate and enfold their molluscan prey. Physiological aspects of this expansion are reported in the present paper, and in the succeeding one (Russell-Hunter and Apley, 1968), the existence of a pedal water-sinus system is demonstrated conclusively, and the functioning of the naticid water-sinus system is discussed.

In his recent, and otherwise excellent, review of locomotion in molluscs, Morton (1964) largely dismisses the possibility of water uptake being involved in naticid expansion, and notes that no modern workers (over the last eighty years) have claimed to demonstrate any aquiferous system of pedal canals. In the last volume of her fine, comprehensive survey of the invertebrates, Hyman (1967) similarly denies the occurrence of internal spaces for water in the naticid foot. Thus, there is clearly a need for an unequivocal demonstration of the use of a pedal water-sinus system in the expansion of naticids, and it is hoped that the present papers will provide it. However, a brief review of why Morton, Hyman, and others were misled by earlier published accounts, and reached their erroneous conclusions seems required.

Historical Introduction

In the early nineteenth century, a number of workers on the Mollusca believed that expansion of various structures in a wide variety of marine molluscs was due to the direct uptake of sea water into the blood spaces. Molluscan anatomists from the time of Cuvier onwards had clearly described the large number of protrusible or distensible structures: tentacles, proboscides, ctenidia, penes, and the mantle and foot themselves, which structures in typical molluscs contained only *retractor* musculature. Dilation with fluid to turgescence was early recognized as a characteristic method of protraction. Universal theories involving sea-water

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uptake in the mechanisms of pedal dilation in all bivalves and snails were dismissed after a number of workers in the 1880's, but most notably Carrière (1882) and Ray Lankester (1884), had shown that, in many different species in both classes, dilation was achieved by the influx of blood withdrawn from other parts of the body. It remains true that the majority of molluscan organs are dilated by hydraulic mechanisms involving the blood within the spaces of the hemocoel. Carrière (1882) also gives a fairly extensive account of the earlier literature. At almost the same time Schiemenz (1884, 1887) claimed to have demonstrated a system of "aquiferous" tubes in the foot of a naticid snail. His observations were made on the relatively large form, *Polinices josephinus,* and they were undoubtedly accurate as regards both the anatomy and the functioning of the system. Schiemenz had earlier been, with Carrière and Ray Lankester, among the group of malacologists strongly opposed to the more general theories of sea-water uptake, but he chose to work on a representative of what now appears to be the *only* group of gastropods to have a water-sinus system—the family Naticidae. As might be expected, the generalization of hemal distension was widely accepted, and Schiemenz's exceptional case ignored or even denied (see, for example, such general surveys of the Mollusca as those of Lankester, 1883, 1891; Simroth, 1879, 1896–1907; and Pelseneer, 1906).

It should be remembered that earlier workers on the mechanics of movements in soft-bodied animals were sometimes confused as to the physiological capabilities of muscle. Simroth (1879), for example, even invoked "active extension" of muscles in discussing molluscan mechanics. The fact that muscles in *all* animals can exert force *only by contracting* had been established by the beginning of this century. The concept of antagonistic sets of muscles acting around a hydrostatic or hydraulic skeleton in soft-bodied animals became clarified, and an early classic account of the molluscan hemocoel as the "force-transmitting" skeleton acted upon by antagonistic muscles is that of Biederman (1905).

Later workers on snail pedal anatomy and mechanics (among them; Weber, 1924; Rotarides, 1934; Lissmann, 1945a, 1945b) emphasized the importance of the hemal hydraulic skeleton. Perhaps the first worker since Schiemenz to investigate problems of dilation in a naticid was Morris (1950) who, working on the smallish Australian species, *Uber strangei,* produced rather inconclusive evidence of water uptake, and claimed that partial expansion was possible in air. A group of unrelated gastropods where the foot undergoes turgid hypertrophy encompasses the South African species of *Bullia* (family Nassariidae), whose ecology closely parallels that of the naticid genus *Polinices.* Once thought to involve water uptake (Gilchrist, 1916), modern investigation of pedal expansion in *Bullia* (Brown and Turner, 1962; Brown, 1964), using such methods as radiography with the blood loaded with radio-opaque dyes, has shown the dilation to be due to blood movement alone. In the monograph which incorporates their extensive and detailed synthesis of existing knowledge of prosobranch snails, Fretter and Graham (1962) mention the work of Schiemenz, but note that in the smaller British naticid species, *Natica catena* and *N. alderi,* they could find no pedal water pores. In his excellent book on the mechanical and evolutionary aspects of body-cavities in metazoans, Clark (1964) discusses locomotion in naticids, but regards the foot in all gastropods as consisting of erectile tissue, dilated by blood. Thus Morton (1964) in his review was perhaps justified in dismissing water uptake as a feature of naticid expansion, and in stating, "No worker has verified Schiemenz's canals. . . ."

The present work was carried out at Woods Hole at different times in the summers from 1961 to 1966. The existence of a pedal water-sinus system in the naticid, *Polinices duplicatus,* and the use of inulin-labelled sea water in its investigation, were briefly reported (Russell Hunter and Apley, 1965) in a note on temporary hyperthermia in that species. While the present results were being prepared for publication, Bernard (1968) has independently demonstrated uptake into a pedal water-sinus system in the West-Coast species, *Polinices lewisi,* though with weighing experiments involving small numbers of narcotized snails, without direct records of volumes of water involved, and without labelling experiments. The present paper reports the results of extensive weighing experiments on "trained" un-narcotized snails, including the relation of different degrees of expansion to the water volumes involved, and the construction of "balance sheet" equations involving the mass of the shell and its actual capacity. The succeeding paper (Russell-Hunter and Apley, 1968) describes and discusses the use of inulin-labelled sea water in the investigation of the relation of the various water spaces and of the rates of water exchange. A third paper is being prepared on the micro-anatomy and histology of the naticid pedal water-sinus system.

Materials and Methods

On anatomical grounds, the family Naticidae (in the superfamily Naticacea) is placed in the suborder or order Mesogastropoda (formerly the Taenioglossa). It is worth noting that most other familiar families of predaceous gastropods using protrusible proboscides, including the Muricidae (e.g. *Urosalpinx*), Buccinidae, Nassariidae, and Conidae, differ anatomically from the naticids and are classified in the suborder or order Neogastropoda (formerly the Stenoglossa). Most work was done on the two commonest species of naticids at Cape Cod, *Polinices duplicatus* and *Lunatia heros,* collected on tidal flats at West Barnstable Harbor, at Orleans Town Pond, and at Duxbury. *P. duplicatus* survives lowered salinities and high temperatures, is the better adapted littoral species and is abundant in certain localities (Russell Hunter and Grant, 1966). *L. heros* achieves the largest size of local naticids, is less resistant, and is essentially a sublittoral species colonizing the intertidal in certain areas. A few observations have also been made on four smaller species, *P. immaculatus, L. triseriata, Natica clausa,* and *N. alderi.* All experimental snails were marked with individual numbers (using various colors of model "dopes" and nail varnishes) and specimens of *P. duplicatus* were maintained in healthy condition in laboratory aquaria for over two months.

The present experiments all depend on the fact that it proved possible to habituate individuals of the two larger naticid species to handling. After a few hours of training, a snail would remain expanded on being transferred to a dry aluminum weighing tray to be weighed in air. There was considerable individual variation in responses and in retention of responsiveness even among snails of similar size.

A triple-beam, and subsequently a torsion balance, were used for the weighings (all to the nearest 0.1 g.). An engineer's dial caliper was used to measure the shells—the measurement recorded here as "shell length" being actually the greatest transverse diameter of the globose shell, from the outer edge of the peristome to the opposite periphery of the last whorl of the shell. The closed volumes of shells were determined by displacement weighings, each cleaned shell being filled with plaster of paris.

In the majority of weighing experiments a numbered snail, of known shell length and contracted weight, was allowed to expand. It was then transferred

TABLE I

Pedal expansion and retraction in Polinices duplicatus: weights in air, and expelled water volumes

Snail #	Shell length mm.	Contracted wt. g.	Estimated degree expansion	Expanded wt. g.	Weight difference g.	Actual water expelled ml.	Expansion Index
d1	61.0	106.2	7	261.6	155.4	136.3	246
d2	35.0	14.7	8	40.0	25.3	22.9	272
d3	40.9	24.7	5	53.8	29.1	29.2	218
d6	26.7	5.9	4	10.9	5.0	5.2	185
d7	52.2	46.0	10	170.2	124.2	111.7	370
d10	44.2	32.1	10	174.2	142.1	134.2	543
d12	36.8	16.9	10	52.8	35.9	34.3	312
d13	36.2	15.8	9	52.6	36.8	35.0	333
d16	33.2	12.0	7	29.7	17.7	17.2	248
d18	36.9	17.7	8	55.8	38.1	35.7	315
d19	33.6	12.8	8	31.7	18.9	18.6	248
d20	41.9	23.6	9	71.8	48.2	45.8	304
d22	35.9	14.4	5	27.9	13.5	13.6	194
d24	16.5	1.8	9	5.2	3.4	2.5	289
d27	49.6	45.1	5	86.3	41.2	40.6	191
d28	46.1	39.8	6	94.6	54.8	54.2	238
d31	43.5	33.7	5	58.9	25.2	25.3	175
d32	41.5	24.6	7	62.8	38.2	38.2	255
d35	44.8	35.0	6	76.8	41.8	41.6	219
d36	46.1	37.7	8	85.7	48.0	46.3	227
d41	41.2	26.6	7	69.0	42.4	41.1	259
d42	39.8	25.0	6	58.0	33.0	33.1	232
d44	52.5	49.7	9	154.3	104.6	104.5	310
d45	47.4	39.6	9	123.7	84.1	84.0	312
d46	46.9	33.4	10	142.8	109.4	109.2	428
d47	43.3	30.5	10	128.1	97.6	92.4	420
d49	46.6	33.4	8	90.4	57.0	57.5	271
d51	41.7	25.3	9	69.6	44.3	44.3	275
d201	22.8	3.9	5	7.4	3.5	3.5	190
d207	30.4	9.8	9	31.2	21.4	21.3	318
d209	48.1	35.6	9	121.9	86.3	86.3	342
d212	46.2	33.1	8	105.4	72.3	73.8	318
d213	43.6	26.9	9	79.2	52.3	52.4	294
d217	37.9	18.4	10	62.5	44.1	43.8	340
d219	41.3	24.3	10	72.5	48.2	47.6	298
d221	43.3	28.6	10	92.3	63.7	62.4	323
d223	29.0	8.6	8	24.3	15.7	15.9	283
d226	34.6	14.5	8	39.8	25.3	25.5	274
d228	41.2	22.5	10	70.7	48.2	46.7	314
d229	36.3	15.0	10	79.8	64.8	64.0	532
d232	44.3	31.9	5	66.5	34.6	34.3	208
d272	43.4	25.0	8	76.7	51.7	49.7	307

TABLE II

Repeated pedal expansion and contraction in Polinices duplicatus

Snail #	Shell length mm.	Dates measured	Contracted wt. g.	Estimated degree expansion	Expanded wt. g.	Weight difference g.	Actual water expelled ml.	Expansion Index
d4	39.8	June 28	21.5	5	39.6	18.1	19.1	184
		June 30	21.5	7	49.6	28.1	23.1	231
		June 30	21.5	8	57.2	35.7	35.6	266
		Aug. 2	20.6	8	54.0	33.4	33.3	262
d5	47.8	June 29	39.9	8	110.7	70.8	67.0	277
		July 1	39.9	6	84.9	45.0	42.8	213
		July 1	39.9	7	85.0	44.9	45.6	213
		Aug. 2	37.3	6	82.2	44.9	44.9	220
d8	47.5	July 3	35.9	10	123.8	87.9	75.9	345
		Aug. 2	34.8	9	128.3	93.5	93.6	369
d50	44.5	July 14	29.8	8	90.4	60.6	58.9	303
		Aug. 1	30.5	10	114.9	84.4	84.3	377
d210	47.5	July 8	36.8	7	101.2	64.4	63.5	275
		Aug. 20	35.0	8	107.6	72.6	71.7	307
d270	51.1	Aug. 21	40.4	9	127.9	87.5	87.2	317
		Aug. 21	40.4	5	93.2	52.8	50.9	231
		Aug. 21	40.4	7	108.0	67.6	66.0	267

to a dry weighing tray, weighed "damp-dried" in air and then forced to contract. The volume of water discharged into the tray was measured, and then the contracted snail weighed again in air. Other experiments involved different sequences.

RESULTS

Preliminary trials soon demonstrated that the weight difference between the snail expanded and contracted was nearly always equal to the volume of water expelled on contraction. This was true for differing degrees of expansion, and for both *Polinices duplicatus* and *Lunatia heros.* Individual variation was less marked in smaller snails, which tended to expand more rapidly after repeated expansions and forced contractions. Medium-sized (shell lengths 30–45 mm.) and larger (45–70 mm.) snails mostly tended to sulk after a third or fourth forced contraction. However, about a third of the medium-sized snails continued to re-expand relatively rapidly, after ten forced contractions within four hours. Such snails are responsible for a disproportionate amount of the data in this paper, and similar snails were deliberately chosen for the inulin-loading experiments (Russell-Hunter and Apley, 1968).

The main series of weighing experiments involved 91 specimens of *Polinices duplicatus* and 22 of *Lunatia heros* in 188 and 47 cycles of weighings, respectively. Table I presents results for 42 typical specimen cycles of *P. duplicatus,* and Table II shows some results of weighings repeated at intervals of up to six weeks.

It proved necessary to set up an arbitrary scale (1–10) for the degrees of expansion achieved. Only the visually estimated degrees of expansion from 4 to 10 were actually employed. Snails remaining considerably less than half-expanded were rated—4, snails half-expanded—5, two-thirds expanded—7, three-quarters expanded—9, and "considerably more than three-quarters expanded" and fully expanded—10. Ratings 6 and 8 were intermediates between the more definable 5, 7 and 9, and the subjectivity of the assessment resulted in relatively more snails being assessed in these two intermediate grades. Such estimates of degrees of expansion are shown in Tables I and II.

The last column of both tables shows a calculated expansion index, which is $M_E/M_C \times 100$ when M_E is the expanded weight and M_C the contracted weight. In Figure 1 expansion indices calculated from 188 cycles (91 specimens) for *Polinices duplicatus* are plotted against the estimates of expansion. Frequencies of index numbers are shown as histograms, at each estimated degree of expansion, and for clarity the calculated index numbers are grouped in class intervals of five units (*i.e.,* index numbers from 243 to 247 inclusive are given the class mark 245). There is fair agreement between the visual assessment and the index calculated from the ratio of weights.

Similar results were obtained with the 22 specimens of *Lunatia heros* which were habituated to handling. "Hypersensitive," and therefore untrainable, individuals were more common in this species. Specimens of *L. heros* of from 34.2 to 70.0 mm. shell length had contracted weights ranging from 13.6 g. to 133.3 g., and gave expanded weights from 24.5 g. to 319.6 g. The assessed degrees of expansion (over 47 experimental weighing cycles) mostly lay in the 5 to 7 range, and most of the corresponding calculated index numbers lay between 185 and 225, the highest index of expansion for the species being 262. It is interesting that index numbers can be calculated similarly for the 20 specimens of *Polinices lewisi* whose contracted and expanded weights are reported by Bernard (1968). These would average only 180, the highest index number achieved being about 202. The specimens of *P. lewisi* used were mostly large (mean contracted weight = 149 g.) and therefore comparable to the largest *L. heros* reported on above. Thus, to date, the most reproducible results from laboratory weighings—and the largest recorded expansion indices—have been derived from the more euryoecic, and more readily "trainable," species, *Polinices duplicatus*.

Significantly, observations on smaller naticid species were disappointing. Pedal expansion and retraction have been observed in *Polinices immaculatus, Lunatia triseriata* and *Natica clausa* from the Cape Cod area, and in *Natica alderi* from Scotland. In all four species, retraction is a more rapid process than in *P. duplicatus* and *L. heros,* and habituation to handling well-nigh impossible. The only one of these smaller species in which appreciable discharge of water on contraction could be detected was *L. triseriata.* Measurement was difficult: the largest specimens had shell lengths of 12.2 mm., and contracted weights of about 0.41 g. Assessment of the weight (or volume) of water involved in expansion in *L. triseriata* is hampered by proportionately large variations in the amounts carried on the surface of the snail. However, with no claims to accuracy, only about 0.25 ml. uptake is involved in large specimens of *L. triseriata.* These disappointing data may help to explain the inconclusive evidence gained from Morris's

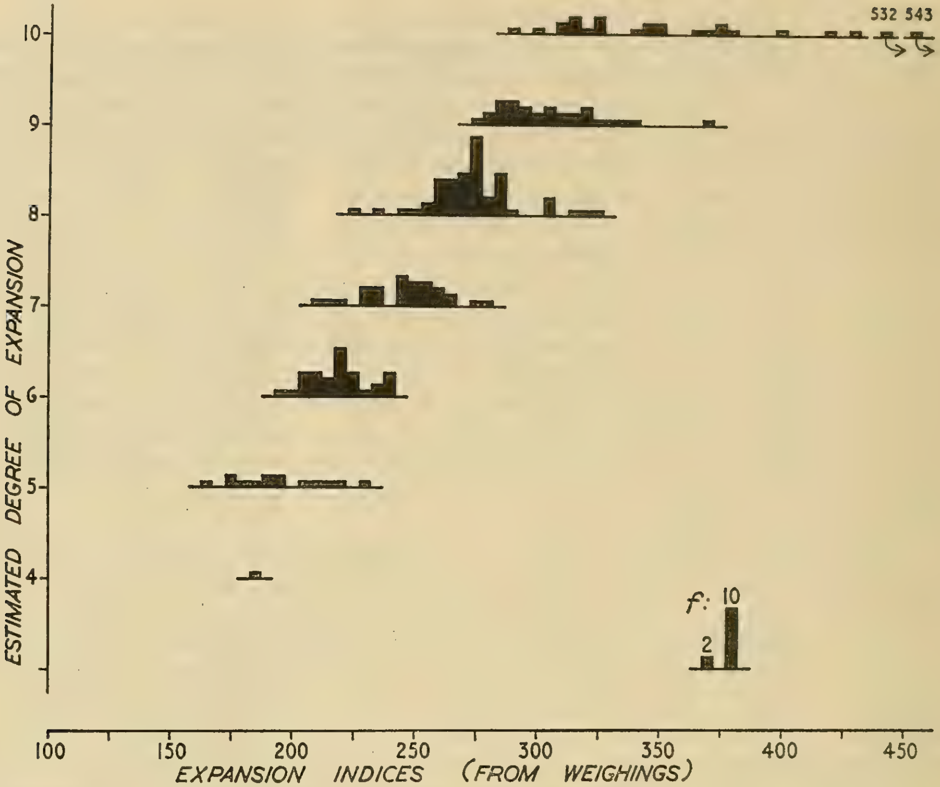

FIGURE 1. The relation of visually estimated degrees of expansion in the naticid, *Polinices duplicatus*, to the expansion indices calculated from the results of weighing contracted and expanded snails. Results derived in 188 weighing cycles from 91 individual snails are shown as histogram frequencies of index numbers. For clarity the calculated index numbers have been grouped in class intervals of five units. For further discussion see text.

(1950) study on the medium-sized Australian species, *Uber strangei*, where water uptake for most specimens must lie in the range 4–7 ml. However, results of weighing experiments on specimens of *Polinices duplicatus* as small as *Uber* were consistent and conclusive (for example, see snails d24 and d201 in Table I).

Some observations on the methods and *rates* of expansion and contraction in *Polinices duplicatus* are worth noting here. (Extensive records of the times involved in expansion were also made during the inulin-loading work—see Russell-Hunter and Apley, 1968.) Retraction can be rapid, particularly in non-habituated specimens newly brought from the field and, in such cases, can be accomplished in 2.5–4.0 seconds (medium-sized *P. duplicatus*). During such rapid contraction, the characteristic "jets" of sea water from the marginal pores of the water-sinus system along the sole edges of the mesopodium are seen. Experimentally forced contractions in "trained" snails usually take longer, and can take 10–15 seconds in medium-sized specimens that have been habituated to handling. During such slow "reluctant" contractions it is clear that almost all water (excepting that from the mantle-cavity which is expelled through the *inhalant* siphon) is discharged *via* the mesopodium and its marginal pores; successive waves of muscular contraction pass over both the "plowshare" of the propodium, and the propodial and metapodial folds surrounding the shell, driving the water contained in their water-sinuses into those of the mesopodial system and thence to the exterior. An account of the anatomy and histology of the system is being prepared, but a few notes on general functional morphology may be required here. Naticids retain to a great extent the three-fold division of the primitive molluscan foot. The propodium is large, wedge-shaped when expanded, and is the plowshare of locomotion (cutting the characteristic canal-shaped—rectangular in cross-section—tracks on the surface of sand flats). With the mesopodium, it is also the organ used for enveloping the prey and manipulating it into the correct position for the proboscis to go to work. [In *Polinices duplicatus* and *Lunatia heros,* this does not necessarily mean positioning the bivalve's umbones at the mid-anterior end of the propodium ready for boring, though this is more usual. Both species consume small and medium-sized razor-clams, *Ensis directus,* at Barnstable and elsewhere, without boring, by positioning the anterioventral gape of the razor-clam for proboscis action.] The posterior edge of the propodium is directed upwards in the "propodial shield" which envelopes the anterior third of the shell when expanded. The shield also encloses the head and mantle edge, including the inhalant pallial siphon and exhalant fold, and is responsible for molding the characteristic egg-masses or sand-collars (actually shaped between the propodial shield and the shell). The mesopodium is the largest part of the foot, provides the locomotory sole and is capable of great lateral expansion in enfolding prey and in some kinds of locomotion. There are no upwardly directed folds of the mesopodium. The metapodium is archetypically the upper posterior division of the foot, last to be withdrawn in gastropod veligers after torsion, and bearing the operculum. In naticids, the metapodium when expanded conceals the operculum while its folds, shaped like a thick cup, enclose most of the posterior two-thirds of the shell. The metapodial folds have been termed epipodial, but this term might be better restricted to upwardly directed folds of the mesopodium (which in some archeogastropods give rise to characteristic sensory structures), and it is certainly incorrect (on comparative grounds, see Fretter and Graham, 1962) to term *all* the pedal structures, apart from the propodium, either "metapodium" as does Morris (1950), or "postpodium" as does Bernard (1968).

Thus the great bulk of the water discharged on retraction, and taken up more slowly during expansion, passes through the marginal pores and major water-sinuses of the mesopodium. The experiments using inulin-labelled sea water (Russell-Hunter and Apley, 1968) have shown that only about 5–7% of the volume of sea water taken up on expansion is taken in through the pallial siphon to the mantle-cavity, and only about 2% of the increased weight on expansion is due to superficial water on and between the shell and expanded surfaces. Thus about 90% of the water uptake first enters the mesopodial water-sinuses and then is pumped by contraction of the major mesopodial columnar muscle bundles into the water-sinuses of the propodium and metapodium, the marginal pores being first sealed by contraction of the smaller distal vertical muscles at the mesopodial edges. All the muscle columns are ensheathed by the lining of the water-sinuses, consisting of a very thin pavement epithelium facing the lumina underlain by a tough layer of collagen-like connective tissue. [The water-sinuses are completely separate from the circulatory system: arterioles and branch sinuses of the hemocoel run with and through the muscle columns. Claims that blood can be involved in the fluids expelled on naticid contraction (Morris, 1950) could only be based on damaged specimens subjected to highly stressed and uncoordinated retraction. Schiemenz (1884, 1887) described the sheaths separating the water-sinuses from the tissues, and his observations are confirmed by recent work (Bernard, 1968; Russell-Hunter, in preparation).]

Normal expansion in healthy specimens of *Polinices duplicatus* takes from 3 to 8 minutes, and somewhat longer in *Lunatia heros*. Initial uptake through the minute marginal pores (about thirty on each side) depends partly on the ciliation of the epithelia around their openings but more on the "recoil" elasticity of the connective tissue sheath system. The latter forces are almost certainly augmented by the temporarily increased blood pressure *within* the muscle columns. The relative times taken in contraction and re-expansion are closely analogous to those for sea-anemones where, similarly, muscles cause contraction, and ciliary and elastic forces are responsible for expansion (Batham and Pantin, 1950). Second and successive experimental expansions within a few hours are possible in *Polinices,* but in habituated specimens the time taken in re-expansion progressively increases (mean times: for first re-expansion in a day's experiments—6.2 minutes, for fourth re-expansion within three hours—21.5 minutes).

In air, on dry surfaces, the larger naticids *cannot* re-expand. The columellar muscle can relax, which allows the operculum to gape, revealing the retracted metapodial folds and the "crumpled" mass of the mesopodium. Only when the latter is in contact with water can real expansion take place. Some experiments with medium-sized, contracted specimens of *P. duplicatus,* set up in shallow water in large weighed petri dishes, showed that expansion was possible with water depths of 3.5–4.0 mm., provided the contracted shell was placed so that the mesopodial margin came in contact with the water when the operculum gaped. Such experiments involved relatively slow expansions (13–26 minutes), and the visually estimated degrees of expansion would be 4 or 5 rather than 9 or 10, but the weight gained by each snail closely matched the water removed from the dish. Only in deeper water does full expansion occur, and only if nearly completely submerged is the mantle-cavity filled and functional. Other experimental investigations of water uptake using dyes such as phenol red were superseded by the experiments with inulin-labelled sea water reported in the next paper (Russell-Hunter and Apley, 1968).

Both in the laboratory and under field conditions, the larger naticids remain continuously expanded for many days (even weeks). The sea water expelled on the first contraction of freshly collected specimens has contained unidentified ciliate protozoans on eight occasions. From two large specimens of *Polinices duplicatus,* first discharged water samples yielded 7 and 3 living harpacticoid copepods, respectively. These were identified as *Tisbe gracilis* by Dr. Harry C. Yeatman, to whom our thanks are due. Harpacticoids have been expelled on five further occasions and have included all ages from nauplii to females with egg-sacs.

The last results to be discussed here are based on the data which were accumulated on the actual shell weights and contained volumes for *Polinices duplicatus*. The weight of the dry shell plus operculum (W) in 69 individuals of shell length (L) from 6.2 mm. to 60.3 mm. (mean = 27.6 mm.), ranged from 0.057 g to 38.7 g. with a mean of 7.09 g. Ratios of W/L^3 calculated for the 69 snails (W in g., L in cm.) give a mean value of 0.166. The total volume (V) of the filled shell (by displacement) in 61 individuals of shell length from 6.2 mm. to 56.8 mm. (mean = 27.3 mm.), ranged from 0.074 ml. to 58.8 ml. with a mean of 9.96 ml. Ratios of V/L^3 calculated for the 61 snails give a mean value of 0.253. These two sets of figures include smaller snails, while the habituated experimental animals are all medium or large (the 48 snails of Tables I and II have shell lengths ranging from 16.5 to 61.0 mm., with a mean of 41.3 mm.).

It is perhaps worth using such volume figures to dismiss another erroneous theory of naticid expansion. This theory, discussed by Morris (1950) and Brown and Turner (1962), suggests that naticid pedal expansion could result from compensatory water being taken to spaces "around the animal, within the shell." Of course, Brown (1964) has shown that pedal expansion in the nassariid, *Bullia*, is accomplished entirely by movement of blood into foot, sea water flowing in to fill a "free space" between the visceral tissues and the shell. In an expanded *Bullia*, the volume of sea water contained within the shell, but outside the tissues, has been estimated at about twice the volume of water in the mantle-cavity, or up to 80% of the shell's volume (Brown, 1964). Nothing like this is possible in a large naticid. For example, experimental snail d209 (Table I) with a shell length of 48.1 mm. would have a calculated total shell volume of 28.2 ml. This is compatible with the recorded contracted weight of 35.6 g., and implies a (retracted) capacity of about 20 ml. for tissues, blood, and water. This capacity should be contrasted with the volume difference recorded (Table I) between the expanded and contracted states of 86.3 ml. Thus, application of a "free space" theory to the facts of naticid expansion is absurd.

The shell volume and weight figures can be used in "balance sheet" equations.

If T is the mass in g. of the contained tissues and retained water in the contracted snail, then: $T = M_C - W$.

Also, if the tissues have a s.g. near 1.0, T can be regarded as an approximately equivalent volume in ml. and, if V_S is the volume of shell material in ml., then: $T = V - V_S$.

Re-arranging these we have: $M_C = W + V - V_S$. But the s.g. of the shell is approximately 2.57 (calcite s.g. = 2.71), so that: $V_S = W/2.57$ and: $M_C = W + V - (W/2.57)$. Values assessed above of $W = 0.166L^3$ and $V = 0.253L^3$, allow us to predict the contracted weight in terms of the shell length: $M_C = 0.354L^3$. The actual contracted weights of the 91 specimens of *Polinices duplicatus* which provided the data for Figure 1 are fitted very well by this curve. Predicted values can be extended to expanded snails since the expansion index: $I = M_E/M_C \times 100$, and thus: $M_E = I \times 0.354L^3/100$ and, for example, for estimated expansions of 8 with an $I = 270$, then: $M_E = 0.956L^3$. Predicted curves based on shell length for each level of expansion can be made in this way and provide good fits for the 188 actual expanded weights on which Figure 1 is based. There is obviously an element of syllogism in such predictions of M_E, but the above predicted curve of M_C does *not* involve the actual data on contracted weights. Further, the broad agreement of both sides in such "balance sheets" shows that no major weight factor has been omitted in this consideration of the relevance of weighing experiments to the mechanics of expansion in *Polinices.*

Discussion

The historical introduction to this paper discusses how the mechanism of pedal expansion in naticid snails, correctly explained by Schiemenz (1884, 1887), was ignored or denied in the literature for eighty years. It is hoped that the present papers will provide the needed unequivocal evidence of the functioning of the pedal water-sinus system in naticids. The simple weighing experiments have already been discussed in the perspective of the differing water uptake involved in different degrees of expansion, in relation to the times involved in contraction and expansion, and in the relation to the weight and volume values for different parts of the snail. The essential equivalence of the weight loss on contraction to the sea water expelled, and the fit of actual contracted and expanded weights to values calculated in relation to shell length, are stressed. The relation of various water spaces, and the rates of water exchange are discussed elsewhere (Russell-Hunter and Apley, 1968).

There remain to be discussed certain mechanical and evolutionary aspects of the naticid water-sinus system.

The majority of movements in molluscs involve transmission of forces generated by muscle contractions through the hydraulic skeleton provided by the blood in the hemal meshwork of the molluscan hemocoel. In a large number of cases, molluscan organs are dilated and extended rather slowly by influx of blood, and are withdrawn relatively fast by intrinsic retractor muscles. The underlying anatomical pattern of obvious retractors within each structure without obvious antagonists locally placed is characteristic (Russell-Hunter, 1968). This reliance on distant antagonists, along with the unchanging total blood volume in the hemocoelic hydraulic skeleton, together are responsible for many of the peculiar features in the mechanics of molluscan locomotion and other movements. For example, limitations arise in the number of extensile structures which can be dilated and protruded at one time. If a pulmonate snail is observed in copulation, the sensory and locomotory organs of the head and foot are flaccid and crumpled, a large proportion of the blood volume being involved in dilation of the genital structures. Further, in most gastropods and bivalves the total blood volume is limited to that which can be withdrawn along with the tissues into the closed shell. To a limited extent, these difficulties can be bypassed by compartmentalization of the hemocoel. Certain mechanically efficient molluscan organs where this is the case have been investigated. Capacity to seal off a potentially variable amount of blood in an organ allows the use of intrinsic muscles in local functional antagonism. Examples include the use of radial muscles to extend the siphons (by "thinning" the siphonal walls) in tellinid clams (Chapman and Newell, 1956), and the radial arrangement of antagonists to the main propulsive circular muscles in the mantle wall of cephalopods. In both cases the fluid content of the wall can be isolated from the rest of the molluscan hemocoel and this allows the efficient use of local antagonistic muscles.

The adaptive significance of the great capacity for pedal expansion in naticids is obvious, and has already been discussed in relation to both locomotion and predation. Mechanically it is based on the capacity for water uptake into internal spaces to provide a hydraulic skeleton of variable total volume. Significantly the volume of the hydraulic skeleton of the pedal water-sinus system is not limited (like hemal volume) by the capacity of the closed shell. Other molluscs, notably bivalves with fused mantle edges and massive siphons, make use of contained sea water in a hydrostatic skeleton of variable volume. In forms like *Mya* and *Hiatella,* closure of the mantle openings allows the siphonal musculature to act as the antagonist of the shell adductor muscles around a temporarily constant volume of sea water in the closed mantle-cavity (Russell Hunter, 1949; Chapman and Newell, 1956; Russell Hunter and Grant, 1962). This is the basis of the boring mechanism in *Hiatella* (Russell Hunter, 1949), and of the step-wise process of siphonal extension in *Mya* (Chapman and Newell, 1956). Of course, the potential of the water-sinus system in naticids, where the total capacity of the hydraulic skeleton can be three or four times the capacity of the shell, is much greater than that of the myacean bivalve's mantle-cavity.

Compartmentalization is also important in the naticid hydraulic skeleton. During expansion, the muscular "pumping" which transfers sea water from the mesopodial water-sinuses to those of the propodium can be followed by a period of further uptake through the mesopodial pores. During this process the already dilated and turgid propodium must be sealed off from the flaccid "absorbing" mesopodium. Occasional inefficiencies in unhealthy or tired snails can be revealing. Normally the first stage of retraction is a rapid transfer of most of the propodial water to the mesopodium before columellar contraction. Occasionally, the "sphincter" muscles between propodium and mesopodium remain contracted (as they are phasically during expansion) when the columellar muscle has already begun to retract the head-foot. Rather rarely, a turgid propodium is trapped against the shell edge and injury results. More frequently, metapodial folds are trapped between operculum and shell. In these cases, the initial retraction is interrupted, the columellar muscle relaxes temporarily, the operculum gapes a little, the metapodium is withdrawn properly, and the retraction is completed. Another, more frequent, inefficiency occurs during expansion when the propodium is being filled by contraction of the mesopodial muscles. Normally, all the mesopodial pores are first sealed but, occasionally, some parts of the mesopodial margin remain relaxed and outward jets of water can be detected with dyes or suspended particles during the filling of the propodium. Apart from the overall capacity for propodial isolation, parts of the anterior water-sinus system must themselves be operable as small "closed" hydraulic units with local sets of antagonistic muscles. The evidence for this comes from behavioral, rather than mechanical, observations. Part of the propodium in *Polinices duplicatus* can wrap around and pick up a small bivalve, then transfer it across or back to another temporary propodial fold, rotate the clam, and reverse it end for end. The propodium can then manipulate it into the "correct" position for the proboscis, and form the two folds of the "vice" which holds the clam during boring. The manipulations involved in catching an active medium-sized *Ensis* can be even more elaborate. It should be remembered that the principal sensory organ in the functionally blind naticids is also the propodium.

(The greatly reduced eyes are "buried" behind the propodial shield in the expanded snail.) Observations of a *Polinices* "searching" for prey with the extended propodial tip "scanning" the sand surface like an elephant's trunk, or of the capture and handling of an *Ensis,* emphasize the adaptational significance of the disproportionate pedal expansion. There is even a report of "swimming" using undulating movements of the propodium in *P. josephinus.* Most of the activities of the larger naticids *depend* on the mechanical properties of the pedal water-sinus system.

As with many biological problems, it is easier to discuss the adaptational significance of the pedal water-sinus system, than to speculate on its evolutionary origins. The latter is made additionally difficult by the phyletically isolated position of the Naticidae within the taenioglossan mesogastropods. They represent a line which has evolved the structures and habits of predaceous carnivores (like those of stenoglossans) completely independently. The suggestion by Cox (1960) that naticids could be allied to that aberrant group of archeogastropods, the Neritacea, is not supported by the evidence of functional anatomy (Fretter and Graham, 1962) but does serve to underline the isolated position of the moon-snails. Thus it is almost impossible to set up alleged "relatives" of naticids in which to look for simple versions or precursors of the pedal water-sinus system. Obviously it is worth investigating the elaborate system of the larger naticids as it develops in the young snail, and it may be worth re-investigating the smaller naticids. Some of the latter may prove to possess a simpler, and more than proportionately smaller, water-sinus system.

In spite of the paucity of evidence on evolutionary origins, two hypotheses seem worth advancing. First, in spite of the histological nature of the sinus linings noted above, it seems most likely that the pedal water-sinus system is developed centripetally from an epidermal origin (*i.e.,* is analogous in ontology to nephridia rather than to coelomoducts). Secondly, it seems possible that the system originated as an elaboration of the lumina of anterior or posterior pedal mucous glands like those found in certain mesogastropods, which glands are developed as epidermal invaginations.

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SUMMARY

1. In 1884, Schiemenz published an essentially correct interpretation of the mechanism of pedal expansion in naticid snails. A historical review discusses how this was ignored or denied in the literature for eighty years, and summarizes the need for unequivocal evidence on the problem.

2. Larger naticids, including *Polinices duplicatus* and *Lunatia heros,* have an extensive pedal water-sinus system, and considerable intake of sea water is required for expansion. On dry surfaces, in air, they cannot expand.

3. Naticids can be habituated to handling, and then weighed in air at all degrees of expansion. Extensive weighing experiments show that the sea

water expelled on contraction is always equivalent to the weight difference between the expanded and contracted states. An index of expansion can be calculated relating expanded to contracted weight and this correlates with visual assessments of degree of expansion. Taking contracted weight as 100, half-expanded specimens of *Polinices duplicatus* have index values around 195 and fully expanded ones around 350. For example, this means that a *Polinices* weighing 46 g. contracted, takes in 124 ml. of sea water when it expands fully to a weight of 170 g. Shell weights and capacities can be incorporated in computing predicted curves which fit results from weighing live snails.

4. In healthy medium-sized specimens of *Polinices duplicatus,* retraction takes 2.5–4.0 seconds and expansion takes 3–8 minutes. Retraction is brought about by a sequence of muscle contractions; expansion is largely based on a "recoil" elasticity augmented by local hemal dilation. The larger naticids often remain continuously expanded for many days.

5. The capacity for disproportionate pedal expansion conferred by the water-sinus system is of great adaptive significance to naticid snails—particularly for locomotion in sand and for prey capture. This is discussed in relation to the hemal hydraulic skeleton and the systems of distant antagonists more usual in molluscs. The hypothesis is advanced that the pedal water-sinus system of naticids may have evolved from epidermal invaginations resembling the pedal mucous glands of other mesogastropods.

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PEDAL EXPANSION IN THE NATICID SNAILS. II. LABELLING EXPERIMENTS USING INULIN [1]

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The mechanism involved in the hypertrophic dilation of the foot in naticid snails was first correctly explained by Schiemenz (1884, 1887) as being based upon a system of pedal water-sinuses. The peculiar sea-water uptake involved in naticids was then ignored or completely denied by many writers on molluscan mechanics (Lankester, 1883, 1884, 1891; Simroth, 1896–1907) and this continued until relatively recently (Brown, 1964; Morton, 1964; Hyman, 1967). A review of the history of a nineteenth century theory which erroneously attributed distension and protrusion of all kinds of molluscan organs to direct uptake of sea water is provided by Carrière (1882). Russell-Hunter and Russell-Hunter (1968) give a historical survey of published references to Schiemenz's essentially correct conclusions on naticid expansion, review the reasons which contributed to the denial of his work over eighty years, and summarize the present need (in 1968) for an unequivocal demonstration of the use of a pedal water-sinus system in the expansion of naticids.

The work recorded here and in the preceding paper on the naticid water-sinus system was adumbrated in a brief report (Russell Hunter and Apley, 1965) on temporary hyperthermia in *Polinices duplicatus*. Independently, Bernard (1968) and Russell-Hunter and Russell-Hunter (1968) have now reported weighing experiments which demonstrate the nature and extent of the water uptake involved in pedal expansion in naticids. This second paper describes and discusses the use of inulin-labelled sea water in *Polinices duplicatus* in an investigation of the relation of the different water spaces and the rates of water exchange in expanded snails carried out in summer, 1965. A third paper is being prepared on the microanatomy and histology of the naticid pedal water-sinus system.

Materials and Methods

Brief notes on the systematics and ecology of naticids are given in the preceding paper (Russell-Hunter and Russell-Hunter, 1968). Only one naticid species was used in the inulin-loading experiments, the more euryoecic and more readily "trainable" form, *Polinices duplicatus*. Medium-sized specimens were employed, all collected by hand from a muddy sand flat at Orleans Town Pond, Cape Cod. For each experimental snail, shell size, contracted weight and mean expanded

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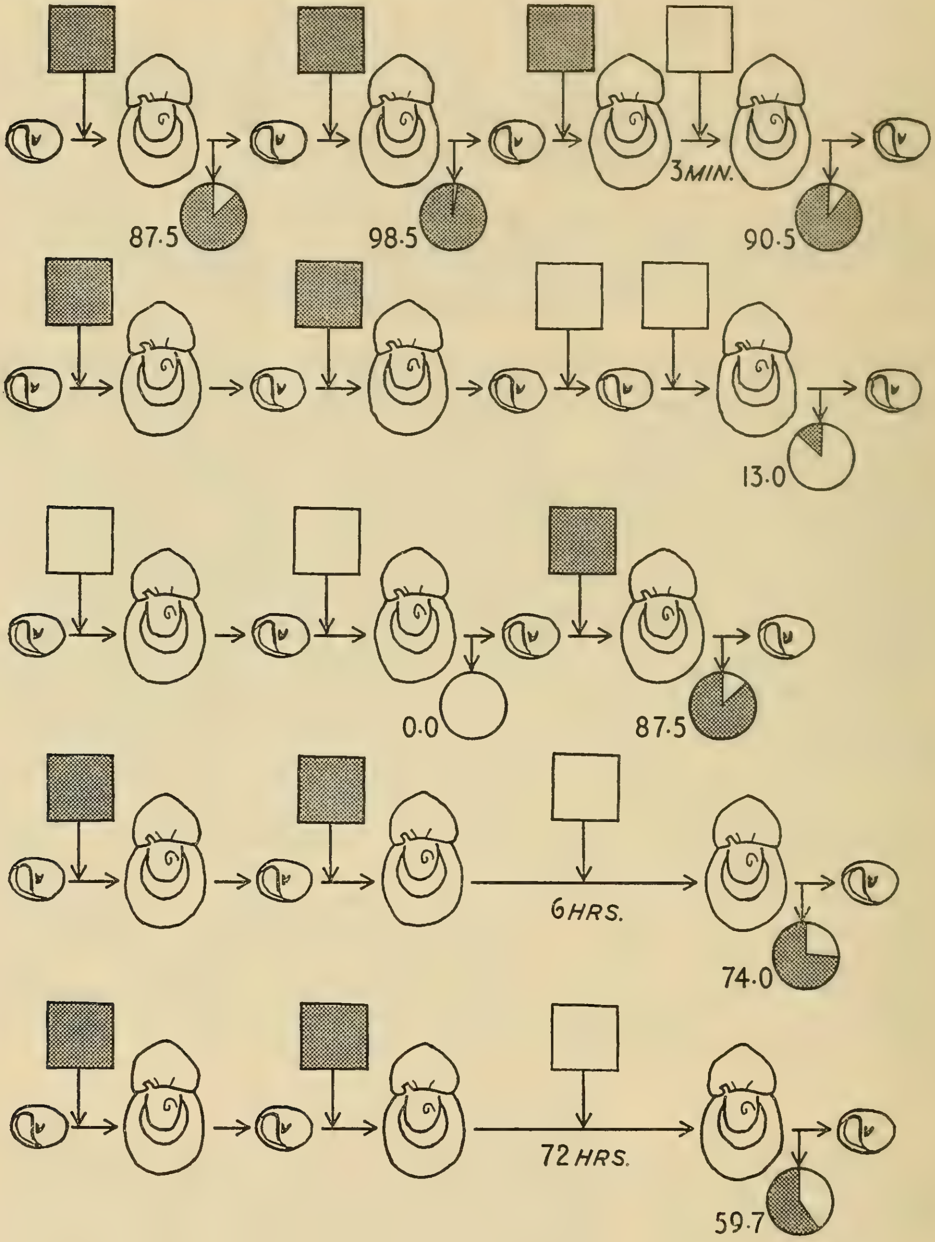

FIGURE 1.

FIGURE 1. Experimental sequences in inulin-labelling of the pedal water-sinus system in the naticid, *Polinices duplicatus*, with average values for some results. The squares above each sequence represent the sea water available for uptake on expansion—shaded squares inulin-labelled, and open squares "clean" sea water; contracted and fully-expanded snails are stylized; and the circles below represent the inulin recovered on forced contraction, expressed as a

weight after training were obtained as in Russell-Hunter and Russell-Hunter (1968). The mean expansion index for each individual allowed a "standard" water uptake to be determined for each, and the actual uptake during an experiment was usually expressed as a percentage of this.

Several sequences were used in inulin-loading, but a typical one would run as follows. A contracted specimen of *Polinices* was placed in a solution of inulin (say 50 mg./l.) in filtered sea water, allowed to expand, forced to contract, allowed to re-expand and then transferred while fully expanded through several washes of clean filtered sea water. It would then be left for a definite time in a known volume of clean sea water, and at the end of the time, weighed in air and forced to contract. Inulin determinations would then be made on the starting solution, the final external sea water, and the internal water expelled at the final forced contraction. This and other sequences are illustrated in Figure 1.

Determinations of inulin concentrations were made by photometric measurement of the red color produced by the reaction of an inulin hydrolysate with resorcinol. Except for minor changes in the preferred range and quantities of samples, this is the method of Schreiner (1950), which in itself is a simplified version of that of Roe *et al.* (1949). Such determinations using resorcinol have been employed by several workers using inulin to determine volumes of extracellular spaces (Ross and Mokotoff, 1951; Cotlove, 1954), and interference from substances likely to be present in biological salines or sea water is minimal. The more cumbersome method using the diphenylamine reaction, employed by some investigators for extracellular volumes (Gaudino and Levitt, 1949), was unsuitable for our purposes.

The analytic procedure (based on Schreiner, 1950) was as follows. Aliquots of 5 ml. of unknown samples or standards were pipetted into 75-ml. Pyrex boiling tubes, each of which already contained 12.5 ml. of 30% hydrochloric acid. Then 5 ml. of an 0.1% solution of resorcinol in 95% alcohol was added to each, and the tubes, loosely stoppered with conical reflux caps, placed in a water bath at 80° C. for 25 minutes. The samples were then cooled in tap water, and the red color determined within 40 minutes. (Trials showed that the absorption values were not significantly changed after 3 hours.) Extinction values were read against a sea-water reagent blank on a Beckman DU spectrophotometer with 1 cm.-path-length cells, mercury lamp and a wave-length setting of 490 mμ. Standards were run with every experiment, as were additional controls (such as samples expelled from the pedal water-sinuses of untreated snails). E-values show a linear relationship to inulin concentrations between 5 mg./l. and 55 mg./l., and experimental procedures were adjusted so that most determinations fell in that range. A stock 0.2% solution of inulin in filtered sea water was prepared at 60° C. (2 g./l.). This was diluted with filtered sea water, immediately before use, to give "exposure"

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percentage of the "loading" concentration. These percentages are also shown as figures to the left of each circle and, in three cases, the average elapsed time while expanded in "clean" sea water is also shown. The first sequence is typical of experiments used in investigation of the effects of "multiple loading" and of "flushing" out of the mantle-cavity, the second and third show two methods of investigation of the residual volume of the water-sinus system in contracted snails, and the fourth and fifth typify experiments on the retention of inulin in the water-sinus system over longer elapsed times while expanded. For further explanation, see text and Tables I–V.

solutions in most cases of approximately 50–60 mg. inulin/l. Such high starting concentrations in the experiments with *Polinices* allowed much greater accuracy in the later determinations of inulin in wash waters and in samples from diluted pedal water. Most results are expressed both as concentrations of inulin in mg./l. and as percentages of the (subsequently determined) starting concentration.

RESULTS

In a group of preliminary experiments, numbered and trained specimens of *Polinices duplicatus* (with contracted weights ranging from 17.3 g. to 26.8 g.) were transferred, while contracted, into inulin solutions (46 mg./l. or 97 mg./l.) where they were allowed to expand fully. Thus the water taken into the pedal water-sinuses was inulin-labelled. They were then gently transferred, without much contraction, through successive washes in large volumes of "clean" sea water for periods of from 17 to 34 minutes. After weighing, damp-dried, in air (expanded weights 42.8–67.0 g.), forced contraction yielded water samples (23.5 to 40.6 ml.) from the pedal water-sinuses. These had inulin concentrations ranging from 42.4% to 52.6% of the appropriate starting levels.

TABLE I

Inulin concentrations in the pedal water-sinuses of Polinices duplicatus immediately after loading (i. e., less than 1 second washing before force contraction and sampling)

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./l.		Number of loading cycles	Final concentration as percentage of initial uptake
			Initial uptake	Final sample		
III	R2	26.4	60.0	58.3	3L	97.2
VC	RR8	25.6	63.3	63.0	2L	99.5
VC	PP2	23.8	63.3	61.8	2L	97.6
VC	PP4	29.0	63.3	62.7	2L	99.1
VC	PP9	20.3	63.3	62.8	2L	99.2

This reduction to about half of the starting concentration of inulin in the water recovered from the snails on contraction, obviously could involve several diluting factors. These include: first, residual water present in the contracted water-sinuses before exposure to inulin; secondly, the water contained in the mantle-cavity and rapidly exchanged by the ciliary respiratory currents during the washes in clean sea water; and thirdly, water exchanged between washing baths and the water-sinuses because of small partial contractions and re-expansions during the transfer process. Many of the later experiments were designed to distinguish among these three factors and, as might be expected, the first two proved to be relatively constant for each individual *Polinices,* while the third varied greatly (though contributing least dilution to the results in the best habituated snails).

Before turning to these more complex experiments and to those extending over longer periods of time, it is worth noting that even this preliminary series of results from "single loadings" with inulin is important in confirming the existence of closed spaces containing sea water in the expanded naticid. Recent weighing

TABLE II

Multiple inulin loading of the pedal water-sinuses in Polinices duplicatus, followed by thorough flushing of the mantle-cavity

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./1.		Number of loading cycles	Final concentration as percentage of initial uptake	Maximum mantle-cavity volume as percentage of water-spaces
			Initial uptake	Final sample			
IIA	R1	23.9	58.3	53.1	4L	91.1	8.9
IIA	R5	16.4	58.3	53.8	4L	92.3	7.7
IIA	P3	26.3	58.3	53.7	4L	92.1	7.9
IIC	R5	16.4	58.3	52.0	4L	89.2	10.8
III	R1	23.9	60.0	57.1	4L	95.2	4.8

experiments on naticids (Bernard, 1968; Russell-Hunter and Russell-Hunter, 1968) merely belatedly confirm and extend Schiemenz's (1884, 1887) findings. The demonstration that a large quantity of inulin-labelled sea water can be carried in the expanded naticid through "clean" wash waters and even through air in a "damp-dried" snail, is a different *kind* of unequivocal evidence for the functioning of the water-sinus system.

In the majority of the 108 successful inulin trials run, the question of the residual volume of the pedal water-sinuses in the contracted snail could be bypassed by multiple loading. In other words the snail was allowed to expand in inulin-labelled sea water, force contracted, then allowed to expand again in the same solution and so on. Initially, three and four successive loadings were carried

TABLE III

Residual volume of the pedal water-sinuses in contracted Polinices duplicatus by two methods

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./1.		Number of loading cycles	Final concentration as percentage of initial uptake	Residual volume as percentage expanded water-sinus volume
			Initial uptake	Final sample			
VIIC	RR6	36.1	66.8	9.8	2L + 1SW	14.7	14.7*
VIIC	RR1	42.1	66.8	8.8	2L + 1SW	13.2	13.2*
VIIC	PP7	24.8	66.8	8.3	2L + 1SW	12.4	12.4*
VIIC	PP6	24.8	66.8	7.4	2L + 1SW	11.1	11.1*
VIIC	PP2	23.8	66.8	9.0	2L + 1SW	13.5	13.5*
VIIA	PP8	22.5	66.8	59.4	1L	88.9	11.1†
VIIA	PP5	23.5	66.8	57.7	1L	86.4	13.6†
VIIA	PP3	19.5	66.8	59.1	1L	88.5	11.5†
VIIA	PP2	23.8	66.8	57.6	1L	86.2	13.8†

* The first group of five snails were loaded twice with inulin-labelled sea water, force contracted, washed and allowed to re-expand in "clean" sea water before force contracting for the final sample.

† The second group of four snails were taken from clean sea water, force contracted, loaded once with inulin-labelled sea water and rapidly washed in "clean" sea water before force contracting for the final sample.

TABLE IV

Inulin retention in the pedal water-sinuses of Polinices duplicatus after multiple loading and elapsed times from 5 to 20 hours

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./l.		Number of loading cycles	Elapsed time (hrs. mins.)	Final concentration as percentage of initial uptake
			Initial uptake	Final sample			
VIII	RR1	42.1	61.8	39.8	2L	5.15	64.4
VIII	RR5	35.8	61.8	49.3	2L	5.30	79.8
VIII	RR6	36.1	61.8	50.1	2L	5.31	81.1
VIII	PP8	22.5	61.8	41.5	2L	6.03	67.2
VIII	PP1	28.5	61.8	48.6	2L	6.33	78.6
VB	RR4	36.1	59.6	43.3	3L	12.02	72.7
VB	PP7	24.8	59.6	39.3	3L	12.06	65.9
VB	RR2	41.2	59.6	38.2	2L	12.17	64.1
VA	RR3	30.3	59.9	36.9	3L	20.57	61.6
VA	RR9	24.7	59.9	37.6	4L	20.58	62.8
VA	RR5	35.8	59.9	40.6	4L	21.12	67.8
VA	RR7	33.8	61.1	42.0	4L	21.23	68.7

out, but it became clear that two successive loadings would suffice to label the residual volume (at least within the limits of accuracy imposed by other variables). In Table I are presented five typical results of sampling immediately after multiple loading with only a brief (less than 1 second) wash to remove surface contamination. As can be seen the water expelled from the pedal water-sinuses in these cases has inulin concentrations between 97.2 and 99.5% of the initial uptake. Thus superficial water on the shell and expanded surfaces can only amount to to 0.5–2.8% of the increase in weight of an expanded *Polinices*. In the experiments of Table II, multiple loading was followed by washing, extending over several minutes, in large volumes of sea water. The five cases presented are typical of snails where no contraction or major pedal movement took place during

TABLE V

Inulin retention in the pedal water-sinuses of Polinices duplicatus after longer elapsed times (2–3 days)

Experiment series #	Snail #	Contracted weight (g.)	Inulin concentration mg./l.		Number of loading cycles	Elapsed time (hrs. mins.)	Final concentration as percentage of initial uptake
			Initial uptake	Final sample			
VIB	RR9	24.7	63.1	48.3	2L	58.13	76.5
VIB	PP9	20.3	63.1	40.8	2L	58.11	64.7
VIB	RR8	25.6	63.1	31.2	2L	58.35	49.4
VIB	RR3	30.3	63.1	49.8	2L	58.32	78.9
VIA	PP4	29.0	66.8	33.8	2L	72.15	50.6
VIA	RR7	33.8	66.8	32.8	2L	72.19	49.1
VIA	RR4	36.1	66.8	45.7	2L	72.36	68.5
VIA	RR2	41.2	66.8	47.2	2L	72.34	70.7

the manipulation, and therefore the final sample concentrations reflect dilution by the proportion of water in the mantle-cavity which has had time to become thoroughly flushed out by the ciliary currents. Therefore, the maximum percentage of all water spaces in the expanded snail made up by the pallial cavity lies between 4.8 and 10.8%. Other circumstantial evidence, and an average value of 2% for superficial water, would suggest that for medium-sized *Polinices duplicatus,* mantle-cavity water usually makes from 5 to 7% of the volume taken in on expansion.

The factor of the residual pedal water-sinus volume (when the snail is contracted) is assessed in two ways in Table III. (See also the procedural sequences in Figure 1.) The first group of results in Table III is derived from snails loaded twice with inulin, force contracted and washed while withdrawn, and then allowed to re-expand in "clean" sea water. Thereafter, force contraction yields a water sample from the pedal water-sinuses with an inulin concentration proportional to the residual volume of the water-sinuses when the snail was contracted. The residual volumes thus represent 11.2–14.7% of the volume of the water-sinuses in the fully expanded snail. Another approach was to load snails once only, rapidly wash and then force contract for a water sample. In this case the dilution of the final inulin concentration is proportional to the residual space. Final concentrations of 86.2–88.9% imply residual volumes of 11.1–13.8% by this method—a satisfactory concordance.

As noted above, the majority of experiments began with multiple loading so the potential dilution from the residual volume of the contracted water-sinus (mean value 12.8%) can be ignored. However, all the other experiments on inulin retention in the water-sinuses after various elapsed times can be regarded as showing a basic dilution of up to about 10% resulting from the combined pallial and superficial volumes of water. Superimposed on this are the major differences in water exchange resulting from individual variations in sensitivity and activity which cause different degrees of pedal retraction within the period of the experiment.

Table IV presents some data on inulin retention in snails 6, 12 or 20 hours after transfer to "clean" sea water. Of the water within the pedal water-sinuses, 64.4–81.1% can remain unexchanged for 5–6 hours, and 61.6–68.7% for 21 hours. It should be realized that this set of results represents a biased group, because any snail which has responded to any stimulus by retraction during the 21 hours has been automatically removed from the experimental series.

This bias is even more true of the results on inulin retention over 2–3 days which are presented in Table V. The data were gained from our best habituated snails, and considerable precautions had to be taken to maintain the animals in conditions free from major changes in light, temperature, tactile and vibrational stimuli during the 58 and 72 hours of the experiment. It is remarkable that 49.1–70.7% of the pedal water could remain unexchanged after 72 hours. Other circumstantial evidence, from commensals (Russell-Hunter and Russell-Hunter, 1968), from the occurrence of temporary hyperthermia (Russell Hunter and Apley, 1965), and from other behavioral data, confirms the potentially static nature of the water after it has been taken up into the pedal water-sinuses. It seems likely that, in nature, a large naticid can remain continuously expanded for periods of weeks.

Discussion

Weighing experiments have been used to demonstrate the nature and extent of sea water uptake into the pedal water-sinus system during the expansion of naticids. The experiments with inulin-labelled sea water reported in the present paper provide additional evidence, particularly as regards the relation of the various water spaces and as regards the rates of water exchange. Both topics merit further discussion.

The experiments showed that there are three components in the volume of sea water responsible for the weight increase in expanded snails. Only about 2% is superficial water on the snail's shell and expanded surfaces, and the water rapidly circulating through the mantle-cavity amounts to a further 5–7% of the total. Approximately 90% of the sea-water uptake on expansion enters the pedal water-sinus system. The earlier conclusion that this system is completely separated from the blood in the hemocoelic spaces (Schiemenz, 1887; Russell-Hunter and Russell-Hunter, 1968), is confirmed by the figures for recovery of inulin in many experiments. In other words, there is no dilution of the sea water in the water-sinus system that is not accounted for, and none that could involve exchange with other body fluids. A residual volume of sea water is retained in the contracted water-sinus system and, immediately after contraction, this amounts to 12.8% of the volume of the water-sinuses in the expanded snail. Once again, the evidence from the inulin-loading experiments shows that there is no exchange between the sea water of this residual space and the blood at any time. Similarly, while the snail remains contracted there is no exchange between the residual space and the environmental sea water outside the animal.

Exchange between the environment and the water-sinuses of a fully expanded snail takes place only to the extent that partial contraction followed by re-expansion occurs. (Over a considerable number of "less successful" experiments not fully reported above, there was good correlation between the degree of dilution of the inulin load and the observed frequency and extent of partial contractions.) The data from inulin-labelling prove unequivocally that the pores of the mesopodium remain continuously closed in fully expanded specimens of *Polinices duplicatus*. As noted in the discussion of the preceding paper (Russell-Hunter and Russell-Hunter, 1968), the mechanical value of the pedal sea water to the snail depends on it being closed off from the environment as a hydraulic skeleton (temporarily of constant volume) capable of transmitting forces between antagonistic muscles. It is still remarkable that inulin-labelling showed that 49–71% of the pedal sea water could remain unexchanged after 72 hours.

At first sight, it would seem that surprisingly "stagnant" nature of the water in the pedal water-sinuses would present certain physiological problems to the snail. This does not seem to be so, though questions such as long-term osmotic stress have not yet been investigated. More can be said on the problems of internal temperature and oxygenation. The large static water content, sealed off from the environment, can result in a condition of temporary hyperthermia in *Polinices* (Russell Hunter and Apley, 1965)—a most unusual condition in a marine "poikilothermic" animal. This was detected using thermistor probes in field and laboratory. Snails crawling on exposed tidal flats on a summer's day had internal temperatures ranging from 2.0° C. to above 0.3° C. below the temperature of the upper 1 cm. of the sand. The temperature of incoming tidal water could be as much as 6.5° C. below the temperature of the sand-flats, and the snails could remain hyperthermic for an appreciable time after tidal submergence. Subjected to lowered temperatures of this order, larger snails (fully expanded weights of 102–159 g.) remained hyperthermic to the extent of 1.8° C. after 5 minutes, 0.9° C. after 10 minutes, and 0.1° C. after 45–60 minutes. Russell Hunter and Apley (1965) note that, empirically, heat is retained about as well as in an equivalent volume of confined but circulating water, but markedly less than in equidimensional foam latex rubber. Such comparison implies some exchange of water *within* the animal during cooling but no exchange with the outside. This is completely in accord with the other evidence on the use of the contained sea water as a hydraulic skeleton. Such a temporary state of hyperthermia has little metabolic importance but is probably of considerable behavioral significance since littoral moon-snails become active immediately after their inundation by the rising tide.

The other physiological question concerns the oxygenation of this "stagnant" mass of internal sea water. There is no evidence to suggest that the water has a lower oxygen tension than that of the snail's blood in the adjacent sinuses. There is circumstantial evidence to the contrary provided by the protistan and harpacticoid commensals which have been recovered from expelled water (Russell-Hunter and Russell-Hunter, 1968). Further, even the most isolated tissues of animals fully expanded for many days remain healthy and responsive. In other words, the ensheathing pavement epithelium and collagen-like connective tissue layers, while mechanically tough, offer no physiological barrier to the diffusion of oxygen.

In broad terms, it seems as if very little energy is expended by a *Polinices* in remaining fully expanded, and perhaps there is a more considerable energy expenditure involved in a complete cycle of contraction and re-expansion. It would seem that the capacity for habituation, which we have exploited in these experiments, must have some adaptive significance. Further, it is clear that sustained contraction and handling over a period of minutes will produce a traumatic change in behavior in *Polinices duplicatus*. "Wild" snails which have experienced such a forced contraction re-expand within 8 minutes, but soon burrow deep into the substratum where they remain immobile but expanded for some time. This behavior was detected as introducing a source of bias when capture-recapture methods were being used to assess population density in *Polinices* (Russell Hunter and Grant, 1966). This study also produced quantitative evidence that recovery from such trauma is complete in just over two tidal cycles, or in about 25.5 hours.

Finally, it should be noted that although inulin-labelling experiments were carried out only on *Polinices duplicatus*, there is reason to believe that the results would be similar with any *large* naticid. All available information suggests that such features as (1) the large proportion (about 90%) of the sea water uptake going into the pedal water-sinuses, and (2) the potentially static nature of the sea water after it has been taken up, would be as true of *Lunatia heros, Polinices lewisi* and *P. josephinus* as they are of *P. duplicatus*.

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SUMMARY

1. Inulin-labelled sea water has been used in an investigation of the pedal water-sinus system, and other water spaces, in *Polinices duplicatus*. Analyses were by a photometric measurement of the reaction of inulin hydrolysate with resorcinol.

2. Of the sea water uptake during expansion: about 90% enters the pedal water-sinus system, about 5–7% is water which rapidly circulates through the mantle-cavity, and about 2% is superficial water on the snail's shell and expanded surfaces.

3. When the snail is in the contracted state a residual volume of sea water is retained in the pedal water-sinus system, and this can amount to 12.8% of the volume of the pedal system in the expanded snail.

4. There is no exchange between the water-sinus system and the blood at any time and, in the fully expanded snail, little or no exchange between the system and the environmental sea water. Labelling showed that 49–71% of the pedal sea water could remain unexchanged after 72 hours.

5. The surprisingly "stagnant" nature of the sea water in the pedal water-sinuses is discussed. Physiological consequences are probably slight, though, under certain ecological conditions, the large static water content is responsible for an unusual condition of temporary hyperthermia. A hypothesis, that little energy is expended by a *Polinices* in remaining fully expanded, is coupled with evidence of traumatic change in behavior resulting from sustained contraction. It seems likely that the features of water spaces and exchange rates demonstrated in *Polinices duplicatus* would be similar in any large naticid.

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THE IDENTIFICATION AND MEASUREMENT OF SUGARS IN THE BLOOD OF THREE SPECIES OF ATLANTIC CRABS[1]

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Although considerable attention has been given to the physiology and biochemistry of the Crustacea, their carbohydrate metabolism did not become known until Hu (1958) found evidence that it was not unlike that of other animals. Using paper chromatography Hu found glucose, maltose, maltotriose, maltotetrose and two other oligosaccharides in the blood of the crab, *Hemigrapsus nudus*. Since then these same sugars have been reported in *Cancer magister* (Meenakshi and Scheer, 1961) several other crabs (Dean and Vernberg, 1965a), the crayfish, *Orconectes virilis* (McWhinnie and Saller, 1960) and the lobster, *Homarus americanus* (Telford, 1968a). The non-reducing disaccharide, trehalose, was found in nine species of crustaceans by Fairbairn (1958) and in traces in some crabs (Dean and Vernberg, 1965a). In the American lobster trehalose appears to be a significant minor component of the blood involved in the response to handling stress (Telford, 1968b). Several monosaccharides, galactose, mannose, fucose and fructose were found with sporadic occurrence (above references).

In this paper the results of chromatographic analysis of the blood sugars in the crabs *Carcinas maenas, Cancer borealis* and *Cancer irroratus* are related to measurements of blood glucose and total reducing sugar. Some evidence of changes with the molt cycle and reproductive activity is also given. A comparison is then made between these data and the rather scattered observations in the literature.

MATERIALS AND METHODS

Collection of animals and blood samples

Both *C. borealis* and *C. irroratus* were obtained from lobster traps at Port Clyde Maine. Three point five to 4.0 ml. of blood was taken via the articular membrane at the base of the cheliped. Decapod crustaceans become hyperglycemic following the stress of handling (Abramowitz *et al.*, 1944). To avoid this reaction blood samples were obtained at the moment of capture with the animals in as nearly an undisturbed state as possible. *Carcinas maenas* was collected in the intertidal zone at Port Clyde. Only from the largest specimens could 4.0 ml. blood be obtained; normally only about 2.0 ml. were collected. The shore crabs, *C. maenas*, were obtained in several stages of the molt cycle but the other two species were taken only in premolt and late postmolt or intermolt because they do not enter the traps at other times. Collections were made monthly from May through October, 1966, and September, 1967. The size range of *C. borealis* used was from 6 cm. up to about 18 cm. across the carapace. *Cancer irroratus* is smaller and the range used was 5–15 cm. The size range of *C. maenas* was 4–11 cm. In all three species males are generally larger than females.

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As anticoagulant 4.0 ml. Heller's oxalate (Gradwohl, 1943) was used (2.0 ml. for the smaller blood samples). The anticoagulant was evaporated to dryness before use, thus avoiding dilution of the blood sample. The oxalated blood was stored frozen until use.

Preparation of samples

Large amounts of oxalate are necessary because coagulability varies during the molt cycle, presumably because of fluctuating blood calcium levels (Travis, 1955). Excess oxalate, however, interferes with the determination of glucose. Before deproteinizing the 1.0-ml. aliquots of blood, oxalates were precipitated by addition of 2.0 ml. 1% $CaCl_2$. Proteins were precipitated by the Somogyi (1930) method using 2.0 ml. 0.6% NaOH followed by 2.0 ml. 2.2% $ZnSO_4$ (acidified by drop-wise addition of conc. H_2SO_4 so that 1 vol. of zinc sulfate solution exactly neutralized 1 vol. of 0.6% NaOH). After centrifuging a clear supernatant was obtained, representing a 1:7 dilution of the blood. A 1.0-ml. aliquot of this supernatant was diluted 1:4 with distilled water.

TABLE I

Monthly levels of blood glucose and reducing substances (RS) in Cancer borealis (mg./100 ml.) Port Clyde, Maine, May–October, 1966

Month	♂ N	Glucose	(S.E.)	R.S.	(S.E.)	♀ N	Glucose	(S.E.)	R.S.	(S.E.)
May	48	6.8	(0.49)	10.5	(0.55)	49	8.0	(0.62)	10.0	(0.57)
June	47	7.7	(0.38)	12.2	(0.51)	43	6.6	(0.47)	10.3	(0.54)
July	50	8.0	(0.36)	10.5	(0.45)	43	7.6	(0.25)	9.4	(0.30)
August	51	10.6	(0.47)	15.3	(0.55)	52	8.4	(0.50)	11.1	(0.57)
September	53	10.2	(0.69)	12.4	(0.74)	53	10.2	(0.57)	12.8	(0.63)
October	43	9.9	(0.46)	12.9	(0.52)	52	9.5	(0.45)	12.1	(0.48)
September (1967)	58	8.7	(0.47)	13.1	(0.81)	64	8.8	(0.43)	13.0	(0.67)

Determination of glucose and reducing substances

Glucose was determined in 1.0-ml. duplicates of the original Somogyi supernatant using glucose oxidase-o-diansidine ("Glucostat," Worthington Biochemical Corp., Freehold, N. J.) dissolved in 0.02 *M* phosphate buffer, pH 7.0; further details of this procedure have appeared elsewhere (Telford, 1965). Reducing substances were determined in 1.0-ml. duplicates of the diluted Somogyi supernatant (above) using the Folin-Malmros alkaline potassium ferricyanide method (Dische, 1962). In both cases a blank of distilled water and three glucose standards of 5, 10, 20 mg./100 ml. were prepared in exactly the same way as the blood whenever determinations were made.

Paper chromatography

In preparation for chromatography individual blood samples of 1.0 or 2.0 ml. (as available) were coagulated by brief immersion in a boiling water bath (about 45 sec.). The clot was broken up and extracted with three washings of 60% methanol which were then pooled. Deionization on ion exchange columns followed (Telford, 1965) and the eluate was dried at 40° C. The residue was redissolved in 0.1 or 0.2 ml. pyridine (depending on size of original blood sample). Various sugars, alone and in mixtures, were prepared in the same way without showing any changes in chromatographic characteristics. The pyridine solutions were spotted onto Whatman #3 paper and developed for 16 hr. in a descending flow of ethyl acetate:pyridine:water, 8:2:1 (v/v/v) (Jermyn and Isherwood, 1949).

Detection of sugars on the developed chromatograms was by one of three

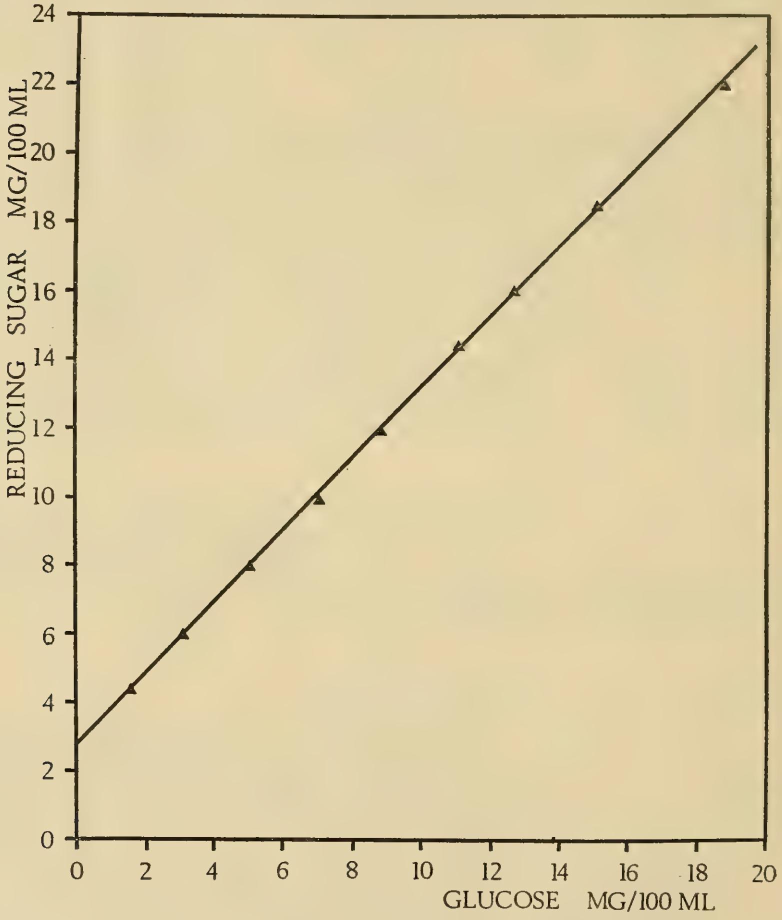

FIGURE 1. Relationship between blood glucose and reducing substances in the crab, *Cancer borealis*.

ways: (1) spray with 3% phthalic acid in 95% ethanol, viewed under UV light, (2) 0.5% benzidine in acetic acid-trichloracetic acid-ethanol, and (3) $AgNO_3$ in acetone followed by 10% NaOH in 80% methanol (all methods in Dawson *et al.*, 1959).

Estimates of concentration in the spots were made by visual comparison with spots of known concentration and by chromatographing various sizes of samples to determine threshold of visibility with the detecting agents. Neither method is particularly accurate but estimates of the right order of magnitude are obtainable.

Reagents

The chemicals used were Fisher "Certified" and the carbohydrates for chromatography were obtained from Sigma Chemical Co. Ltd.

RESULTS

Mean levels of glucose and total reducing substances (RS) in the blood of *C. borealis* made at monthly intervals are given in Table I, together with the number of specimens (N) and the standard error of the mean (S.E.).

TABLE II

Blood glucose and reducing substances during the molt cycle of Carcinas maenas (mg./100 ml.)

Stage	Drach (1939)	N	Glucose	S.E.	Reducing sugar	S.E.
Intermolt	C_4–D_0	23	9.9	(0.81)	13.8	(0.72)
Premolt	D_1–D_4	11	12.2	(1.37)	17.1	(1.41)
Very soft	A_1–A_2	5	5.8	—	7.9	—
Early postmolt	B_1–B_2	2	5.7	—	8.0	—
Postmolt	C_1–C_3	19	8.4	(0.90)	13.5	(1.07)

An analysis of variance indicates that the variation in mean monthly glucose levels (1966) is significant at the 0.05 level. The differences in mean monthly reducing substance levels are generally not significant although a t-test between the July and August levels in males gives a significant result. In spite of appearances the difference between the sexes is not significant.

The individual glucose and reducing substance levels varied quite widely. Glucose ranged from 0.4 mg./100 ml. to 29.3 mg./100 ml. and the range for reducing substances was 3.3–29.5 mg./100 ml. The relationship between glucose and reducing substance is approximately constant. Figure 1 is a plot of mean glucose levels within 2 mg./100 ml. classes against the corresponding mean reducing substance levels. The line has a slope of 1.02 and an intercept of 2.9 mg./100 ml. when glucose is nil. The same values for slope and intercept can be obtained directly. The correlation coefficient between glucose and reducing substances, calculated directly from the 584 paired measurements, is highly significant ($R = 0.914$, $t = 54.22$ with 582 degrees of freedom). The analysis of covariance shows that this relationship between glucose and reducing substances

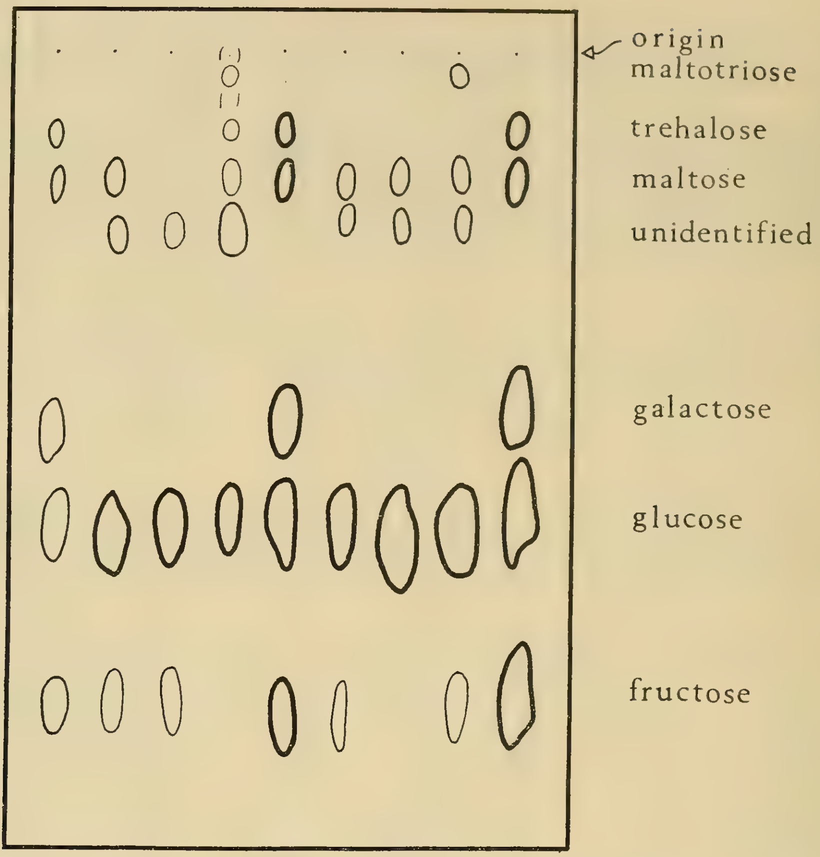

FIGURE 2. Paper chromatogram of blood sugars of *Cancer borealis*. Left, center and right, control mixtures of trehalose, maltose, galactose, glucose and fructose, 1.5 γ, 3.0 γ and 7.5 γ each, respectively (traced from photograph).

does not vary significantly from month to month, nor between the sexes, and it indicates the same level of significance for the variations in monthly mean glucose and reducing substance levels as did the analysis of variance already cited.

The crabs, *C. maenas,* were collected in September, 1967, in various molt stages. Intermolt and very early premolt, $C_4 - D_0$ of Drach (1939), were not separated but were treated together as a single intermolt stage which was recognized by color, hardness of shell, texture, color and consistency of the blood etc. Premolt, stages $D_1 - D_4$ of Drach, was recognized by darker color with blueness of chelipeds, development of new epicuticle and exocuticle and regeneration of damaged appendages. The earliest postmolt stage, stages $A_1 - A_2$ of Drach, was easily recognized by the non-calcified shells and the next postmolt stage, stages $B_1 - B_2$ of Drach, by the partially calcified exoskeleton. Postmolt, $C_1 - C_3$ of Drach, was recognized by the color and texture of the shell and blood. Table II shows blood glucose and reducing sugars in these general molt stages. These differences are found by analysis of variance to be significant at the 0.05 level. No attempt was made to determine the significance, if any, of the differences between sexes, because there are too few observations to produce a reliable result.

A total of 67 specimens of *C. irroratus* were taken from lobster traps in May and June (1966), 31 males and 36 females. The mean glucose level for these was 8.1 mg./100 ml. (S.E. 0.23) and reducing substances 11.9 (S.E. 0.39); no difference in sex was apparent. No other determinations were made on this species (except chromatography, below) because *C. borealis* was readily available in much greater numbers in the areas where lobster traps were being set.

Chromatography of deionized blood samples (Figs. 2 and 3) revealed the occurrence of six sugars in the amounts estimated in Table III. Mobility of the sugars is expressed relative to glucose (Rg) because the solvent front was allowed to leave the paper. The bracketed percentage figure indicates the approximate number of samples in which each sugar was found. The estimated quantity is maximal in these samples. The unidentified carbohydrate was com-

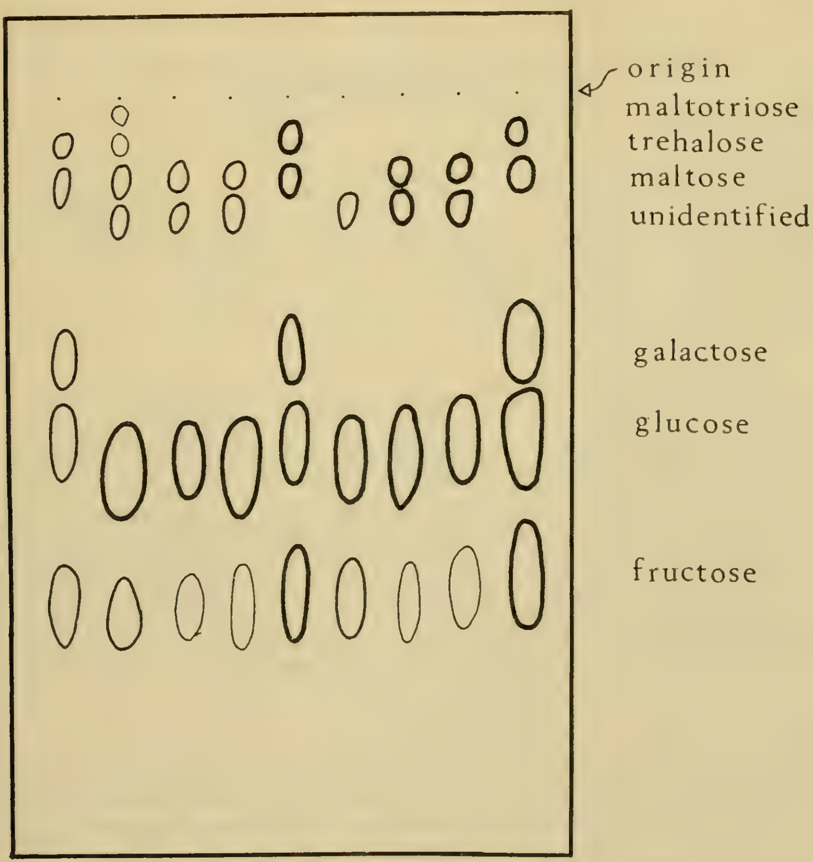

FIGURE 3. Paper chromatogram of blood sugars of *Carcinas macnas*. Controls as in Figure 2 (traced from photograph).

pared to maltose in estimating its concentration. The following criteria were used for identification of the sugars. Maltotriose: plot of $\log_{10}$ Rg against number of hexose units places it on a straight line with maltose and glucose (McWhinne and Saller, 1960) (Fig. 4). No maltotriose was available for direct comparison. Trehalose: same mobility as control spots, reinforcement of spots when trehalose added to sample, lack of reaction with phthalic acid or benzidine indicates non-reducing nature, appearance of spot with silver nitrate at same time as trehalose (this is due to the very strong alkali used, 10% NaOH). Maltose, glucose and fructose: mobilities, lack of proliferation when these sugars were added to blood samples, corresponding blood sugars show reducing activity and appear in same sequence and time intervals with $AgNO_3$-NaOH spray (fructose-glucose-maltose). The mobility of the unidentified component suggests a disaccharide and its reaction with both phthalic acid and benzidine indicates a reducing group.

A total of ten female *C. borealis* was obtained carrying egg masses. On one of these the sponge was old; blood glucose and reducing substances levels were 8.0 and 8.2 mg./100 ml., respectively, in this individual. The respective mean levels for the remaining nine were 12.36 (S.E. 1.18) and 14.47 (S.E. 1.19) mg./100 ml. Twelve females were found with bright orange-red blood and well developed ovaries. These were presumed to be preparing for spawning since the blood color was closely similar to that of the eggs and ovaries and this coloration never appeared in males. Mean glucose and reducing levels were 11.7 (S.E. 1.28) and 12.5 (S.E. 1.17) mg./100 ml. Three pairs of *C. maenas* were captured in copulation and blood was taken from all of the males and two females. No significant difference in blood sugar levels was found despite the ire and pugnacity of the animals (glucose 10.3, reducing substances 13.9 mg./100 ml.).

TABLE III

Estimated concentrations of blood sugars found in three species of crabs with the frequencies of occurrence (%)

Sugar	Rg	*C. borealis*	*C. irroratus*	*C. maenas*
Maltotriose	(0.05)	trace (10%)	trace (30%)	2 mg./100 ml. (30%)
Trehalose	(0.13)	trace (10%)	trace (10%)	1.5 mg./100 ml. (10%)
Maltose	(0.27)	2 mg./100 ml. (70%+)	2 mg./100 ml. (70%)	3 mg./100 ml. (30%)
Unidentified	(0.32)	5 mg./100 ml. (100%)	5 mg./100 ml. (100%)	5 mg./100 ml. (100%)
Glucose	(1.00)	— (100%)	— (100%)	— (100%)
Fructose	(1.40)	1 mg./100 ml. (60%)	1 mg./100 ml. (30%)	1.5 mg./100 ml. (100%)

DISCUSSION

The blood glucose and reducing sugar levels reported here are comparable with those reported previously for decapod crustaceans but some interesting differences emerge. McWhinnie and Saller (1960) found in the crayfish *Orconectes virilis* that glucose levels averaged 3–4 mg./100 ml. and made up about 20–25% of the total reducing substances (by Folin-Wu method). Earlier McWhinnie and Scheer (1958) found the same relationship between glucose and total carbohydrate

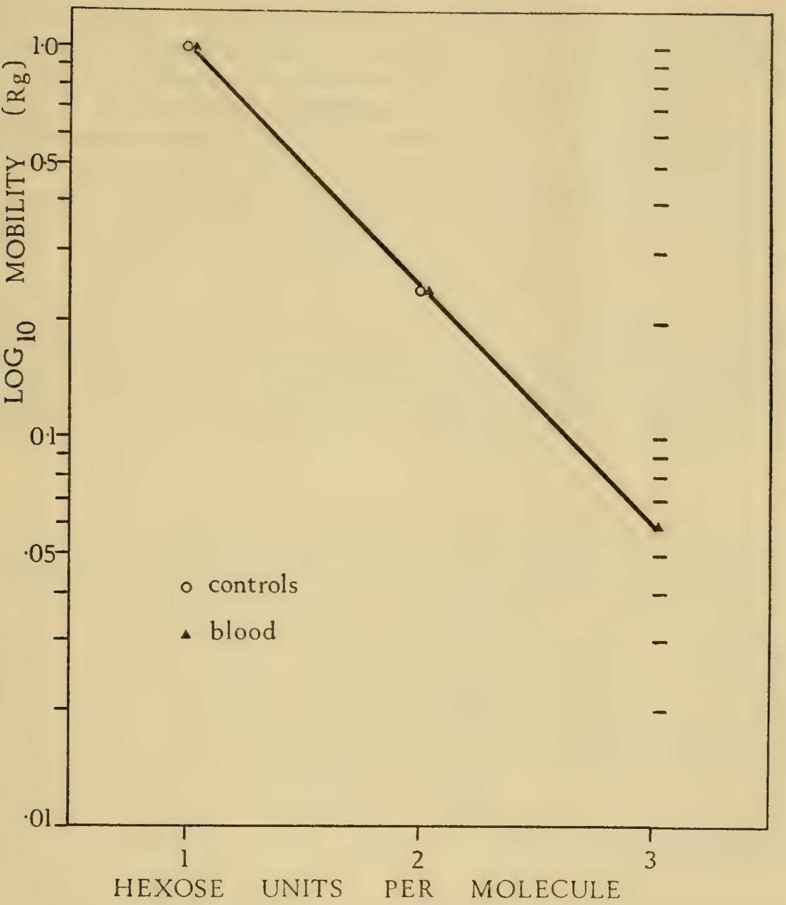

FIGURE 4. Identification of presumed maltotriose by plotting molecular size and $\log_{10}$ mobility (after McWhinnie and Saller, 1960).

(by anthrone method) in the crab *Hemigrapsus nudus.* More recently Dean and Vernberg (1965a, b) using the same methods as McWhinnie and Saller (1960), found the same relationship in several species of crabs and stated specifically that no matter what the glucose level, its ratio to total reducing substances was constant. This is clearly not the case in *C. borealis* where glucose is the variable blood sugar and the non-glucose component remains approximately constant at about 3 mg./100 ml. In the lobster, *Homarus americanus,* this same relationship has been found, variable glucose levels and approximately constant non-glucose around 4.4 mg/100 ml. (Telford, 1968a). In the special case of females carrying eggs there is evidence of significant differences between them and other females (0.01 level by *t*-test). In Table IV these and similar data from Dean and Vernberg (1965b) are compared.

A similar series of changes in glucose levels evidently occurs in the two species but according to the data for *C. sapidus* another reducing substance also undergoes the same changes. No attempt was made to identify this substance. Dean and Vernberg (1965b) appear to have used a heat-coagulated blood for their reducing substance determination. This method does not completely deproteinize the sample and leaves most or all of the non-carbohydrate reducing matter in the filtrate. These interfering substances may have masked the true relationship between glucose and reducing sugars in the studies with heat-coagulated blood.

The amount of color produced by several sugars with the Folin-Malmros method used in this study was determined and expressed as a percentage of the color given by the same amount of glucose. Trehalose, as expected, gave no reaction; maltose gave 60% of its glucose equivalent, fructose 104%. The estimated amounts of sugars other than glucose found in chromatograms (Table III) are the maximum levels found. Assuming that the unknown reducing disaccharide would react like maltose (lactose, for example, gives about 55%), then the total non-glucose carbohydrate component of the reducing substances would have a maximum value of about 6 mg./100 ml. The estimated normal level is closer to 2 mg./100 ml. or, about 60–65% of the non-glucose component. In another study of the lobster, *Homarus americanus*, the non-glucose part of the reducing substances was estimated to be about 50% carbohydrate (Telford, 1968a, 1968b).

TABLE IV

Glucose and reducing substances levels in C. borealis and C. sapidus carrying egg sponges

Species	N	Glucose mg./100 ml.	S.E.	Reducing mg./100 ml.	S.E.	Gluc/Red × 100
Cancer borealis						
no eggs	43	6.59	(0.47)	10.31	(0.55)	64%
new eggs	9	12.36	(1.18)	14.47	(1.19)	85%
old eggs	1	8.00	—	8.20	—	97%
Callinectes sapidus						
no eggs	7	18.47	(2.36)	—	—	20–25%
new eggs	7	37.61	(2.77)	—	—	20–25%
old eggs	9	10.52	(1.03)	—	—	20–25%
C. sapidus data from Dean and Vernberg (1965b)						

In the study of the lobster referred to above a cycle of changes in blood sugar levels during the molt cycle was found in a group of 800 animals. The apparent cycle found here in *C. maenas,* although the number of specimens is much lower, is closely similar. Molting of crabs on the coast of Maine occurs principally in the late summer and fall. The seasonal changes in *C. borealis* probably reflect the molting cycle with glucose increasing in premolt (August-September) and dropping in postmolt. The differences here are not clearly defined because the different molt stages were not separated, the monthly samples being made up of a changing proportion of premolt and other animals. In previous studies such a cycle of changes has not been found although expected (McWhinnie and Scheer, 1958; Scheer, 1959; McWhinnie and Saller, 1960).

As a regular component of crustacean blood fructose has not previously been reported. It was reported as occasionally occurring in *H. americanus* (Telford, 1965; 1968a, 1968b) but it was not found by either McWhinnie and Saller (1960) or Dean and Vernberg (1965a). It has been reported in some insects (Levenbrook, 1950). No sign of the curious "galactan derivative" of McWhinnie and Saller (1960) was found, nor of galactose. Maltotetrose has often been reported by other workers but no spot corresponding with its probable position could be found with the detection reagents used here, nor by examination under UV light when tetroses should fluoresce. Following molting the blood of *C. maenas* has only traces of sugars other than glucose; the oligosaccharides are severely depleted as in lobsters at this stage (Telford, 1968a).

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SUMMARY

1. Blood glucose levels in the crab *Cancer borealis* ranged from 0.4 mg./100 ml. to 29.3 mg./100 ml. with a mean value of 8.6 mg./100 ml. In the same animals blood reducing substances were in the range 3.3–29.5 mg./100 ml. with a mean of 11.6 mg./100 ml. Blood glucose and reducing substances in the other two species of crabs tested, *Cancer irroratus* and *Carcinas maenas,* were in the same ranges.

2. Changes in the blood glucose level account for most of the variations in reducing substances; the other components remain approximately constant, at about 3 mg./100 ml. The relationship between blood glucose and reducing substances is thus a simple straight line one.

3. Variations of blood sugar levels during the molt cycle were found in *Carcinas maenas* and probably occur also in *Cancer borealis.* Qualitative changes in blood sugar composition also occur.

4. Significant changes also occur in *Cancer borealis* females carrying eggs and at this time the relationship between blood glucose and reducing substances changes, the two values converging as the egg mass ages.

5. Paper chromatography of the blood of these three species of crabs shows the presence of glucose, fructose, maltose, an unidentified reducing disaccharide and occasional traces of maltotriose and trehalose.

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INDEX

A

ATPase and actin combining properties of "myosin" from molluscan smooth muscle, 438.
Absorption of lipids by cestode, 80.
Abstracts of papers presented at the Marine Biological Laboratory, 406.
Acclimation, temperature, role of in protein synthesis in liver of toadfish, 130.
Accumulation of blood protein in silkmoth, effect of juvenile hormone on, 466.
Acetate, absorption of by cestode, 80.
Acetylcholine, effects of on tunicate heart pacemaker, 174.
Action of ecdysone in Sarcophaga, 322.
Acropora, photosynthetic pigments of symbionts of, 149.
Adaptive significance of pedal expansion in naticid snails, 548, 563.
Adrenoceptive properties of tunicate heart pacemaker, 174.
Adult male Aedes, sexual responses of, 445.
Aedes, male, sexual responses of, 445.
Akey, D. H., and J. C. Jones. Sexual responses of adult male Aedes using the force-copulation technique, 445.
Algae of coral, pigment of, 141.
Allolobophora, melting temperature of collagen of, 223.
Ambystoma, migratory orientation of, 230.
Amino acid content of lipoprotein from Arbacia eggs, 193.
  incorporation in toadfish liver, 130.
  specificity in bonding of, to tRNA, 181.
Amphibian, migratory orientation of, 230.
  egg, cytochemistry of jelly layers, of, 501.
Anaerobiosis, effect of on ecdysone action, 322.
Anatomy of Polyonyx larvae, 111.
Annelid, development of commensal of, 111.
  fertilization and brood protection in, 208.
  commensal, colony growth of, 96.
Annual Report of the Marine Biological Laboratory, 1.
Annual reproductive cycles in Spisula, 349.
Anophrys as endocommensal of echinoids, 514.
Antheraea, effect of juvenile hormone on synthesis and accumulation of blood protein of, 466.
Apley, M. L. See W. D. Russell-Hunter, 563.
Apter, M. N. See F. M. Child, 416.
Arbacia eggs, lipoprotein from, 193.
Arme, C., and C. P. Read. Studies on membrane transport. II, 80.
Armored dinoflagellates, bioluminescence of, 279.
Arnold, J. M. An analysis of cleavage furrow formation in the egg of Loligo, 413.
  Formation of the first cleavage furrow in a telolecithal egg (Loligo), 408.
Arthropod, reproductive behavior in, 273.
  larvae, effects of diet on, 454.
Ascaris, melting temperature of collagen of, 223.
Ascidian, ecology and growth of, 296.
Asiatic fireflies, fine structure of light organ of, 335.
Atkinson, B. G., and N. A. Granholm. A ciliary activity inhibitor extracted from the nidamental gland of Loligo, 413.
Atropine, effects of on tunicate heart pacemaker, 174.
Ault, C. R. See C. Henley, 422.
Avicularia of ectoprocts, biological role of, 425.
Axial pattern of hydroid colony, 96.
Axon regeneration by cockroach tissues in vitro, 520.

B

Bagley, S. J. See S. Zigman, 443.
Bal, A. K. See P. L. Krupa, 426.
Bang, F. B. See C. L. Reinisch, 432; P. Som, 437.
Bartell, C. K., K. May and M. Fingerman. Electron microscopic examination of components with melanophorotropic activity, 414.
Basis, ultrastructural, for polarotaxis of copepods, 252.
Beauregard, L. J. See J. D. Cassidy, 412.
Behavior, brooding, of Spirorbis, 208.
  reproductive, in Libinia, 273.
  sexual, of Aedes, 445.
  of copepods in polarized light, 239, 252.
Bell, A. L., and G. J. Mpitsos. Morphology of the eye of the flame fringe clam, Lima, 414.
Bennett, M. F., and J. A. Harbottle. The effects of hydrocortisone on the blood of tadpoles and frogs, Rana, 92.

Bennett, M. V. L., and J. P. Trinkaus. Electrical coupling of embryonic cells by way of extracellular space and specialized junctions, 415.
Bennett, M. V. L. See M. E. Kriebel, 426; S. Obara, 430.
Berns, M. W., and W. T. Keeton. Effects of semi-starvation on growth and morphogenesis during the larval stages of a common milliped, Narceus, 454.
Biannual reproductive cycle in Spisula, 349.
Biggaria as endocommensal of Caribbean echinoids, 514.
Biology of fertilization in Spirorbis, 208.
Bioluminescence of dinoflagellate Pyrocystis, 439.
  of dinoflagellates, 279.
  of firefly, light-inhibition of, 476.
  in Obelia, 429.
Bioluminescent proteins, calcium-activated, from ctenophores and hydroids, 422.
Blood, role of in cockroach nerve regeneration, 520.
  content of ecdysone in Sarcophaga, 322.
  of crabs, sugars in, 574.
  of frogs, effects of hydrocortisone on, 92.
  protein in silkmoth, effect of juvenile hormone on synthesis and accumulation of, 466.
Bluefish retina, rhythmic movement of cones in, 530.
Blumenfeld, M., and H. A. Schneiderman. Effect of juvenile hormone on the synthesis and accumulation of a sex-limited blood protein in the Polyphemus silkmoth, 466.
Body size of milliped larvae, effects of diet on, 454.
Bolton, J. R., and R. K. Clayton. Studies with reaction centers from Rhodopseudomonas, 407.
Bonding of amino acids to tRNA, specificity of, 181.
Botryllus, migration of germ cells in, 424.
Brachiopod, larvae of, 263.
Brain coral, pigments of algae of, 141.
Breeding ponds, movements of salamanders near, 230.
Brood protection in Spirorbis, 208.
Buck, J. See M. K. Peterson, 335.
Butyrate, absorption of by cestode, 80.
Byssus threads, factors affecting production of, in Mytilus, 420.

C

Calcified bryozoan, skeletal wall structure of, 436.
Calleja, G. B., G. T. Reynolds and R. J. Podolsky. The use of luminescent systems and image intensification as cytochemical probes, 416.
Campbell, R. D. Colony growth and pattern in the two-tentacled hydroid, Proboscidactyla, 96.
Cancer (crab), sugars in blood of, 574.
Candelas, G. C., S. Temes, J. R. Ortiz and S. Gelfant. Dogfish white blood cells: protein synthesis in vivo and in vitro, 415.
Carbohydrate metabolism in Crustacea, 424.
Carbohydrates in Xenopus egg jelly, 501.
Carbon monoxide secreting tissue of Physalia, fine structure of, 486.
Carcinas, sugars in blood of, 574.
Cardiac physiology of tunicates, 166, 174.
Cardioregulation in Prionotus, 433.
Caribbean sea urchins, endocommensal ciliates of, 514.
Carnay, L. See A. Watanabe, 442.
Carotene in symbionts of corals and clams, 149.
Carotenes in algae of coral, 141.
Case, J., and M. S. Trinkle. Light-inhibition of flashing in the firefly Photuris, 476.
Cassidy, J. D., C. F. Starmer, and L. J. Beauregard. Quantitative autoradiography of vitellogenesis in Artemia, 412.
Cell outgrowth in regenerating cockroach nerve tissue, 520.
  recognition in Asterias, 432.
Ceratium, bioluminescence of, 279.
Cercaria of Cryptocotyle, electron microscopy of excretory vesicle of, 426.
Cerebellar cells of Mustelus, responses of, 440.
Cestode, absorption of lipids by, 80.
Chaetopterus, development of commensal of, 111.
Chasis, S. See W. Troll, 440.
Chen, H. R. See W. S. Vincent, 441.
Cheney, D. P. Red cell morphometrics and volume in the cichlid teleost Tilapia, 105.
Cheney, R. H. See C. C. Speidel, 437.
Child, F. M., and M. N. Apter. Effect of cycloheximide on ciliogenesis, ciliary regeneration and protein synthesis in Arbacia embryos, 416.
Chlorophyll in algae of coral, 141.
  in symbionts of corals and clams, 149.
Cholinoceptive properties of tunicate heart pacemaker, 174.
Chromatophores of Palaemonetes, responses of to adenosine monophosphate, 418.
  of Rhodopseudomonas, reaction centers of, 407.
  in Uca, comparative analysis of control of, 432.

Chromatophoric responses of Uca, variations in, 408.
Chuang, S.-H. The larvae of a discinid (Inarticulata, Brachiopoda), 263.
Cichlid teleost, red cell morphometrics and volume in, 105.
Ciliary activity inhibitor in squid nidamental gland, 413.
  regeneration, effect of cycloheximide on, in Arbacia embryos, 416.
Ciliates, endocommensal, from Caribbean sea urchins, 514.
Ciona heart pacemaker, pharmacology of, 174.
Cirri of discinid larvae, 263.
Claff, C. L., A. Crescenzi and A. J. Landé. Pulsatile pressure extracorporeal blood oxygenation, 409.
Clam, reproductive cycle of, 349.
Clams, photosynthetic pigments of symbionts of, 149.
Clayton, R. K. See J. R. Bolton, 407.
Cleavage furrow in Loligo egg, formation of, 408, 413.
  rate of sea urchin eggs, influence of ploidy and cytoplasmic volume on, 434.
  stages in Arbacia egg, electron microscopy of, 413, 420.
Cockroach, regeneration of tissues of, in vitro, 520.
Codium, pigments of, 141.
Coelenterate, colonial, fine structure of gas-secreting tissue in, 486.
  colony, growth and pattern of, 96.
Cohnilembus as endocommensal of Caribbean echinoids, 514.
Cold-acclimated toadfish, protein synthesis in, 130.
Collagen, melting temperatures of, in relation to body temperature of poikilotherms, 223.
Colonization of Corella, 296.
Colony growth in hydroid, 96.
Commensal crab, larval development of, 111.
  hydroid, colony growth in, 96.
Compensation of liver protein synthesis in temperature-acclimated toadfish, 130.
Composition of low density protein from Arbacia eggs, 193.
  of pigments in algae of coral, 141.
Cones, rhythmic movements of, in retina of bluefish, 530.
Copeland, D. E. Fine structure of the carbon monoxide secreting tissue in the float of Physalia, 486.
Copepods, polarotaxis in, 239, 252.
Copulation of Aedes, 445.
Coral, pigments of, 141.
Corals, photosynthetic pigments of symbionts of, 149.
Corella, ecology and growth of, 296.
Costello, D. P., and H. M. Costello. Immotility and motility of acoel turbellarian spermatozoa, with special reference to Polychoerus, 417.
Costello, D. P. See C. Henley, 422.
Cousineau, G. H. See P. L. Krupa, 426.
Crab, commensal, larval development of, 111.
  reproductive behavior in, 273.
Crabs, sugars in blood of, 574.
Crescenzi, A. A. See C. L. Claff, 409.
Crustacean, larval development of, 111.
  reproductive behavior in, 273.
Current year class Loligo, growth and size distribution of, 366.
Cycle, reproductive, of Spisula, 349.
Cyclidium as endocommensal of Caribbean echinoids, 514.
Cyclops, endogenous rhythm of polarotaxis in, 239.
Cysteinyldopa-iron pigmentation of red species, 419.

D

DNA synthesis in toadfish liver, 130.
Darkness, role of in movement of cones in bluefish retina, 530.
Decapod crustacean, larval development of, 111.
DePhillips, H. A., Jr., and K. E. Van Holde. Oxygen-linked dissociation of hemocyanin. I, 417.
DePhillips, H. A., Jr. See K. E. Van Holde, 441.
Developing elasmobranchs, urea and TMAO levels in, 537.
Development of cockroach nerve regenerates in vitro, 520.
  of Libinia, 273.
  of Polyonyx, 111.
Developmental potentiality of RNA-treated posterior primitive streak of chick embryo, 200.
Diadema, endocommensal ciliates of, 514.
Diadinoxanthin in symbionts of corals and clams, 149.
Diaptomus, polarotactic behavior of, 252.
Diet, effects of on milliped larvae, 454.
Differential interference-contrast microscopy, use of in observation of living squid giant axons, 411.
Digaster, melting temperature of collagen of, 223.
Dinoflagellates, bioluminescence of, 279.
  symbiotic, photosynthetic pigments of, 149.
Dinoxanthin in symbionts of corals and clams, 149.
Diplosoma, growth of, 296.

Discinid, larvae of, 263.
Displacements of migrating salamanders, 230.
Distomum, life-history of, 439.
Distribution and dispersion patterns of Tellina, 419.
  of Loligo in Wood Hole waters, 366.
  of sea water taken up by naticid snails, 563.
Dresden, M. H. Limb regeneration in adult amphibians, 410.
Drugs, effects of on tunicate heart pacemaker, 174.
Dynamics of ecdysone secretion and action in Sarcophaga, 322.

E

Earthworms, melting temperature of collagen of, 223.
Eccles, J. C., H. Taborikova, and N. Tsukahara. Excitation and inhibition of Purkyne cells in the cerebellum of Mustelus, 418.
Eccles, J. C. See N. Tsukahara, 440.
Ecdysis of crabs, in relation to their blood sugars, 574.
Ecdysone, effect of on growth and cuticle formation of Drosophila imaginal discs, 431.
  secretion and action in Sarcophaga, 322.
Echinoderm, feeding and oxygen uptake of, 308.
Echinoderm eggs, lipoprotein from, 193.
Echinoderms, Caribbean, endocommensal ciliates of, 514.
Echinoids, Caribbean, endocommensal ciliates of, 514.
Echinometra, endocommensal ciliates of, 514.
Ecological significance of polarized light sensitivity of copepods, 252.
Ecology of dinoflagellates, 279.
  of Loligo, 366.
  of Malleus and Isognomon, 378.
  of solitary ascidian, 296.
Effects of hydrocortisone on frog blood, 92.
  of semi-starvation on growth of millipeds, 454.
Egg, Xenopus, cytochemistry of jelly envelopes surrounding, 501.
Eggs of Arbacia, lipoprotein from, 193.
Eigner, E. A. See R. B. Loftfield, 181, 428.
Elasmobranch embryos, urea and TMAO levels in, 537.
Elder, H. Y. See R. K. Josephson, 409.
Electrical coupling of embryonic cells, 415.
  stimulation, effects of on action of sulfhydryl reagents in axonal preparations, 423.
Electron microscopy of clam eye, 414.
  of copepod naupliar eye, 252.
  of firefly light organs, 335.
  of gas-secreting tissue in Physalia, 486.
Electroretinogram and spectral sensitivity of scallop, 441.
Elliott, G. F. See R. V. Rice, 433; A. Stracher, 438.
Embryo, chick, development of after RNA treatment, 200.
Embryos, elasmobranch, urea and TMAO levels in, 537.
Endocommensal ciliates from Caribbean sea urchin, 514.
Endogenous rhythm of polarotaxis in copepods, 239.
Environmental changes in light intensity, role of in movement of cones in bluefish retina, 530.
Enzymes, amino acid activating, from various phyla, 181.
Epinephrine, effects of on tunicate heart pacemaker, 174.
Erythrocyte morphometrics in teleost, 105.
Escherichia, amino acid activating enzymes from, 191.
Eucidaris, feeding and oxygen uptake of, 308.
Eurylepta, predation of on Corella, 296.
Expansion of foot in naticid snails, 548, 563.
Eye of bluefish, rhythmic movement of cones in, 530.
  of copepod, fine structure of, 252.
  of dogfish, effect of light on, 443.

F

Facultative self-fertilization in Spirorbis, 208.
Fatty acids, absorption of by cestode, 80.
  in lipoprotein from Arbacia eggs, 193.
Favia, photosynthetic pigments of symbionts of, 149.
  pigment of algae of, 141.
Feeding of tropical sea urchin, 308.
Female spider crabs, reproductive behavior in, 273.
Fertilization-induced increase of trypsin-like activity in sea urchin "membrane," 440.
Fertilization of Spirorbis eggs, 208.
Filamentous protein structures from squid giant axon, 411.
Fine structure of copepod naupliar eye, 252.
  of firefly light organ, 335.
  of gas-secreting tissue in Physalia, 486.
Fingerman, M., R. D. Hammond and R. S. True. The response of the red chromatophores of Palaemonetes to cyclic 3′,5′-adenosine monophosphate, 418.
Fingerman, M. See C. K. Bartell, 414; K. R. Rao, 408, 432.
Fireflies, fine structure of light organ of, 335.
Firefly, light-inhibition of flashing in, 476.

Fish, movement of cones in retina of, 530.
Fisher, F. M., Jr., and R. O. McAlister. A new and improved source of trehalase, 419.
Fisher, F. M., Jr. See M. A. Johnston, 424.
Flashing in firefly, light-inhibition of, 476.
Flesch, P. The cysteinyldopa-iron pigmentation of red species, 419.
Fleshfly, ecdysone secretion and action in, 322.
Float of Physalia, fine structure of, 486.
Foot, expansion of in naticid snails, 548, 563.
Force-copulation of Aedes, 445.
Form and habit in Malleus, 378.
Freeman, S. B. A study of the jelly envelopes surrounding the egg of the amphibian, Xenopus, 501.
Frog blood, effects of hydrocortisone on, 92.
Fructose in blood of crabs, 574.
Functional morphology of naticid snails, 548, 563.
Fungia, photosynthetic pigments of symbionts of, 149.

G

Gametes of Spisula, seasonal changes in, 349.
Ganglion tissue, role of in regeneration of cockroach nerve tissue in vitro, 520.
Gas-secreting tissue in Physalia, fine structure of, 486.
Gastrotrichs, interstitial marine, from Woods Hole region, 423.
Gelfant, S. See G. C. Candelas, 415; T. Pederson, 431.
Genetic variation in nature, distribution of, 409.
Gilbert, W. H. Distribution and dispersion patterns of the dwarf tellin clam, Tellina, 419.
Glaus, K. J. Factors influencing the production of byssus threads in Mytilus, 420.
Glenodinium, bioluminescence of, 279.
Glial cells, role of in cockroach nerve regeneration, 520.
Glucose in crab blood, 574.
Gonad indices of tropical sea urchins, 308.
Gonads of Spisula, seasonal changes in, 349.
Gonopods of milliped larvae, effects of diet on morphology of, 454.
Gonyaulax, bioluminescence of, 279.
Goodenough, D. A., S. Ito and J.-P. Revel. Electron microscopy of early cleavage stages in Arbacia, 420.
Gore, R. H. The larval development of the commensal crab Polyonyx, 111.
Gospodnetić, M. See J. Metuzals, 411.
Granholm, N. A. See B. G. Atkinson, 413.
Green, J. P. See J. R. Stewart, 438.
Green, R. H. See K. D. Hobson, 410.
Greenberg, M. J., R. B. Hill, H. Irisawa and H. Nomura. Pharmacological studies on electromechanical coupling in a phasic molluscan muscle, 421.
Grosch, D. S. Reproductive performance of insects from U. S. Biosatellite II, 408.
Grosch, D. S. See D. F. Moffett, 429.
Grossman, A. See W. Troll, 440.
Growth, colonial, in hydroid, 96.
  of milliped, effects of starvation on, 454.
  of nerve regenerates in vitro, 520.
  and size distribution of current year class Loligo, 366.
  of solitary ascidian, 296.
Gymnodinium, bioluminescence of, 279.

H

Halimeda, pigments of, 141.
Halvorson, H. O. See W. S. Vincent, 441.
Hammer oysters, form and habit in, 378.
Hammond, R. D. See M. Fingerman, 418.
Harbottle, J. A. See M. F. Bennett, 92.
Hart, M. A. The morphology of a mechanoreceptor organ of the bivalve Spisula, 421.
Haschemeyer, A. E. V. Compensation of liver protein synthesis in temperature-acclimated toadfish, Opsanus, 130.
Haschemeyer, A. E. V. Possible role of protein synthetic enzymes in the control of protein synthesis in higher organisms, 406.
Haschemeyer, A. E. V., and J. C. Levy. Determination of rate of polypeptide chain assembly in vivo of Opsanus, 422.
Hastings, J. W., and J. G. Morin. Calcium activated bioluminescent proteins from ctenophore and colonial hydroids, 422.
Hastings, J. W. See J. G. Morin, 429.
Haxo, F. T. See S. W. Jeffrey, 149.
Heart, tunicate, pharmacology of pacemaker of, 174.
  cells, tunicate, pacemaker properties of, 166.
Heart-lung devices, as tested on dogfish and sharks, 409.
Heliopora, photosynthetic pigments of symbionts of, 149.
Hemocyanin dissociation, oxygen-linked, 417.
Henley, C., D. P. Costello and C. R. Ault. Microtubules in the axial filament complexes of acoel turbellarian spermatozoa as revealed by negative staining, 422.
Hill, R. B. The relationship between carbon atom chain length and effectiveness of *n*-alkylamines in inducing rhythmicity of gastropod smooth muscle, 407.
Hill, R. B. See M. J. Greenberg, 421.

Hillman, G. R., and H. G. Mautner. The effect of electrical stimulation on the action of sulfhydryl reagents in axonal preparations, 423.
Hinsch, G. W. Reproductive behavior in the spider crab Libinia, 273.
Hippopus, photosynthetic pigments of symbiont of, 149.
Hirshfield, M. See D. Marsland, 429, 442.
Histology of firefly light organ, 335.
  of Spisula gonads, in relation to season. 349.
Hobson, K. D., and R. H. Green. Asexual and sexual reproduction of Pygospio in Barnstable Harbor, 410.
Hormone, juvenile, effect of on sex-limited blood protein of silkmoth, 466.
  of Sarcophaga, secretion and action of, 322.
Hummon, W. D. Interstitial marine gastrotrichs from Wood Hole. II, 423.
Hydrocortisone, effects of on blood of frog, 92.
Hydroid colony, pattern of growth in, 96.
Hymenolepis, absorptions of lipids by, 80.

I

Identification of sugars in crab blood, 574.
In vitro regeneration of cockroach nerve tissue, 520.
Inarticulata, larvae of, 263.
Inhibition of enzymes or cell division, number of molecules involved in, 428.
  by light of flashing in firefly, 476.
Insect, dynamics of ecdysone action in, 322.
Insemination of virgin female mosquitoes, 445.
Instars of milliped, effects of diet on, 454.
Intensity of light in relation to polarotaxis of copepods, 239.
Interphylogenetic specificity in bonding of amino acids to tRNA, 181.
Inulin-labelling experiments on pedal expansion in naticid snails, 563.
Irisawa, H. See M. J. Greenberg, 421.
Isognomon, form and habit in, 378.
Isolation of low density liproprotein from Arbacia eggs, 193.
Ito, S. See D. A. Goodenough, 420.
Izzard, C. S. Migration of germ cells through successive generations of pallial buds in Botryllus, 424.
Izzard, C. S. See J. Metuzals, 411.

J

Jeffrey, S. W. Pigment composition of Siphonales algae in the brain coral Favia, 141.
Jeffrey, S. W., and F. T. Haxo. Photosynthetic pigments of symbiotic dinoflagellates from corals and clams, 149.
Jelly envelope of Xenopus egg, cytochemistry of, 501.
Johnston, M. A., and F. M. Fisher, Jr. Aspects of carbohydrate metabolism in Crustacea, 424.
Jones, I., and T. E. Rogers. Studies on the endocommensal ciliate fauna of Caribbean sea urchins, 514.
Jones, J. C. See D. H. Akey, 445.
Josephson, R. K., and H. Y. Elder. Rapidly contracting muscles used in sound production by a katydid, 409.
Juvenile hormone, effect of on sex-limited blood protein in silkmoth, 466.
  of Sarcophaga, dynamics of action of, 322.

K

Kaufmann, K. W., Jr. The biological role of Bugula-type avicularia, 425.
Keeton, W. T. See M. W. Berns, 454.
Kelly, M. G. The occurrence of dinoflagellate luminescence at Woods Hole, 279.
Kleinholz, L. H. Crustacean pigmentary-effector isohormones, 425.
Koser, R. See S. Lerman, 428.
Kriebel, M. E. Cholinoceptive and adrenoceptive properties of the tunicate heart pacemaker, 174.
Kriebel, M. E., S. G. Waxman, G. D. Pappas and M. V. L. Bennett, Neurophysiology of oculomotor neurons in teleosts, 426.
Kriebel, M. E. Pacemaker properties of tunicate heart cells, 166.
Krishnakumaran, A. See H. A. Schneiderman, 435.
Krupa, P. L., G. H. Cousineau and A. K. Bal. Electron microscopy of the excretory vesicle in the cercaria of the trematode Cryptocotyle, 426.
Kusano, K., and M. M. LaVail, III. Functionally excitable area of shrimp medullated fiber, 427.

L

Labeled red cells of teleost, morphometrics and volume of, 105.
Labelling experiments on pedal expansion in naticid snails, 563.
Laeospira, fertilization and brood protection in, 208.
Lambert, G. L. The general ecology and growth of a solitary ascidian, Corella, 296.
Landé, A. J. See C. L. Claff, 409.
Larvae of a discinid, 263.
LaVail, M. M., III. See K. Kusano, 427.
Larval development of Polyonyx, 111.
  ecdysone in Sarcophaga, 322.
  millipeds, effects of semi-starvation on, 454.

LASTER, L., AND P. STRITTMATTER. Maltase activity in developing embryos and adult tissues of the surf clam Spisula, 427.
Layers, jelly, of Xenopus egg, cytochemistry of, 501.
LEIKOLA, A. See M. C. NIU, 200.
Lens of dogfish eye, insoluble protein in, 428.
proteins, sulfonation of, 406.
LEOPOLD, R. A. See E. P. MARKS, 520.
LERMAN, S., J. TUTTLE AND R. KOSER. Composition and formation of insoluble protein in the dogfish lens, 428.
Leucocytes of frogs, effects of hydrocortisone on, 92.
Leucophaea, regeneration of tissues of, in vitro, 520.
Levels of urea and TMAO in elasmobranch embryos, 537.
LEVY, J. C. See A. E. V. HASCHEMEYER, 422.
Libinia, reproductive behavior in, 273.
Light, role of in movement of cones in bluefish retina, 530.
-flashing by dinoflagellates, 279.
-inhibition of flashing in firefly, 476.
intensity, in relation to polarotaxis in copepods, 239.
organ fine structure in Asiatic fireflies, 335.
Lipids, absorption of by cestode, 80.
Lipoprotein from Arbacia eggs, 193.
Liver protein synthesis in toadfish, 130.
-RNA-treated chick embryo, development of, 200.
Locomotion of naticid snails, role of pedal expansion in, 548, 563.
LOFTFIELD, R. B., AND E. A. EIGNER. The number of molecules involved in inhibiting either an enzyme or cell division, 428.
LOFTFIELD, R. B., E. A. EIGNER AND J. NOBEL. Inter-phylogenetic specificity in the bonding of amino acids to tRNA, 181.
Loligo, growth and size distribution of current year class of, 366.
LORAND, L. See R. V. MYHRMAN, 430.
Low density lipoprotein from Arbacia eggs, 193.
Luciola, fine structure of light organ of, 335.
Luminescence of dinoflagellates, 279.
of firefly, light-inhibition of, 476.
Luminescent systems and image intensification techniques as cytochemical tools, 416.
Lunatia, pedal expansion in, 548.
Lymphocytes of Tilapia, 105.
Lymphopenia in frog blood after treatment with hydrocortisone, 92.
Lytechinus, endocommensal ciliates of, 514.

M

Macracanthorhynchus, melting temperature of collagen of, 223.
Macrophages of Tilapia, 105.
Male Aedes, sexual responses of, 445.
spider crabs, reproductive behavior in, 273.
Malleus, form and habit in species of, 378.
Maltase activity in adult and embryonic Spisula tissue, 427.
Maltose in blood of crabs, 574.
MARCHIONI, W. W. See B. L. OLLA, 530.
MARKS, E. P., J. P. REINECKE AND R. A. LEOPOLD. Regenerating tissues from the cockroach, Leucophaea: nerve regeneration in vitro, 520.
MARSH, J. B. Isolation and composition of a low density lipoprotein from the eggs of Arbacia, 193.
MARSLAND, D., AND M. HIRSHFIELD. Stabilization of axopodia by D_2O: a pressure-temperature study, 429.
MARSLAND, D. See L. WOLPERT, 442.
MAUTNER, H. G. See G. R. HILLMAN, 423.
MAY, K. See C. K. BARTELL, 414.
MCALISTER, R. O. See F. M. FISHER, 419.
MCPHERSON, B. F. Feeding and oxygen uptake of the tropical sea urchin Eucidaris, 308.
Measurement of sugars in crab blood, 574.
Mechanism of pedal expansion in naticid snails, 548, 563.
Mechanoreceptor organ of Spisula, morphology of, 421.
Melanophorotropic subcellular components, electron microscopic examination of, 414.
Melting temperatures of collagen, as correlated with temperature relationships of poikilotherms, 223.
Membrane transport, 80.
Mercenene, effects of on cell population kinetics, 434.
Metabolism of tropical sea urchin, 308.
Metopus as endocommensal of Caribbean sea urchins, 514.
METUZALS, J. Segregation of filamentous protein structures from the axoplasm of the squid giant nerve fiber by uranyl ions, 411.
METUZALS, J., C. S. IZZARD AND M. GOSPODNETIĆ. Observations of living giant nerve fibers of the squid by differential interference-contrast microscopy, 411.
Microtubules in spermatozoa of acoels, as relater to their motility, 422.
Microvilli in copepod naupliar eye, 252.
Migration, vertical, of copepods, in relation to polarotaxis, 239.
Migratory orientation of Ambystoma, 230.

Milkman, R. On the distribution of genetic variation in nature, 409.
Milkman, R. D. See T. Ohtaki, 322.
Millepora, photosynthetic pigments of symbiont of, 149.
Milliped larvae, effects of semi-starvation on, 454.
Mitochondria in gas-secreting tissue of Physalia, 486.
Moffett, D. F., and D. S. Grosch. Differential effects of LAS detergent on reproductive performance of male and female Artemia, 429.
Mollusc, form and habit in, 378.
  growth and size distribution of current year class of, 366.
  pedal expansion in, 548, 563.
  reproductive cycle of, 349.
Molting in arthropods, control of by steroid hormones, 435.
  of silkmoth, effect of juvenile hormone on, 466.
  stages of crabs, in relation to blood sugars, 574.
Moon-snails, pedal expansion in, 548, 563.
Morin, J. G., G. T. Reynolds and J. W. Hastings. Excitatory physiology and localization of bioluminescence in Obelia, 429.
Morin, J. G. See J. W. Hastings, 422.
Morphogenesis of milliped, effects of semi-starvation on, 454.
  of RNA-treated chick embryo, 200.
Morphology of discinid larvae, 263.
  of hydroid colony, 96.
  of Malleus and Isognomon, 378.
  of Polyonyx larvae, 111.
Mosquito, sexual responses of, 445.
Motor nerve regeneration in cockroach, 520.
Movement of cones in bluefish retina, 530.
Movements of Ambystoma near breeding ponds, 230.
Mpitsos, G. J. See A. L. Bell, 414.
Mucociliary action in nudibranchs, effects of temperature and other factors on, 437.
Mucopolysaccharides in Xenopus egg jelly, 501.
Muscle, phasic molluscan, electromechanical coupling in, 421.
  smooth, induced rhythmicity of, 407.
Myhrman, R. V., and L. Lorand. A continuous rate assay for transpeptidating enzymes, 430.
Myocardium of tunicate heart, pacemaker qualities of, 166.
"Myosin" from molluscan smooth muscle, electron microscopical study of, 433.

N

Narceus larvae, effects of semi-starvation on, 454.
Naticid snails, pedal expansion in, 548, 563.
Naupliar eye of copepod, fine structure of, 252.
Navigation of salamander, 230.
Neo-dinoxanthin in symbionts of corals and clams, 149.
Neo-peridinin in symbionts of corals and clams, 149.
Nerve fiber, functionally excitable area of, 427.
  regeneration by cockroach tissues, 520.
Neurophysiology of oculomotor neurons in teleosts, 426.
Neutrophilia in frog blood after hydrocortisone treatment, 92.
Nicotine-induced alteration of DNA and cell membranes in Arbacia gametes, 437.
Niu, M. C., and A. Leikola. The developmental potentiality of the liver-RNA-treated posterior primitive streak in the chick embryo, 200.
Nobel, J. See R. B. Loftfield, 181.
Nomura, H. See M. J. Greenberg, 421.
Nucleic acid synthesis in dogfish erythrocytes, 431.
Nutrition, role of in development of Polyonyx larvae, 111.
  role of in protein synthesis of toadfish, 130.
  of tropical sea urchins, 308.
Nutritional requirements of larval millipeds, 454.

O

Obara, S., and M. V. L. Bennett. Receptor and generator potentials of ampullae of Lorenzini in the skate, Raja, 430.
Occurrence of bioluminescence in dinoflagellates, 279.
Ocelli of milliped larvae, effects of diet on number of, 454.
Ocellus of copepod, fine structure of, 252.
Ohtaki, T., R. D. Milkman and C. M. Williams. Dynamics of ecdysone secretion and action in the fleshfly Sarcophaga, 322.
Olla, B. L., and W. W. Marchioni. Rhythmic movements of cones in the retina of bluefish held in constant darkness, 530.
Oocyte yolk of silkmoth, effect of juvenile hormone on, 466.
Opsanus, protein synthesis in liver of, 130.
Optical changes associated with nerve excitation and membrane polarization, 442.
Orientation, migratory, of Ambystoma, 230.
  of copepods in polarized light, 239, 252.

ORTIZ, J. R. See G. C. CANDELAS, 415.
Osmotic relations in elasmobranch embryos, 537.
Ostreobium, pigments of, 141.
Ova, Arbacia, lipoprotein from, 193.
Oviducal material surrounding Xenopus egg, cytochemistry of, 501.
Ovum of Xenopus, cytochemistry of jelly layers of, 501.
Oxygen, role of in action of ecdysone, 322.
  -linked dissociation of hemocyanin, 417, 441.
  uptake of tropical sea urchin, 308.

P

Pacemaker properties of tunicate heart cells, 166.
  of tunicate heart, pharmacology of, 174.
PALMER, J. D. See I. SINGER, 436.
PAPPAS, G. D. See M. E. KRIEBEL, 426.
Parasite, absorption of lipids by, 80.
Pattern of growth in hydroid colony, 96.
Patterns of reproductive behavior in Libinia, 273.
Pedal expansion in naticid snails, 548, 563.
PEDERSON, T., AND S. GELFANT. Dogfish erythrocytes: activation of nucleic acid synthesis in a repressed cell, 431.
Peridinin in symbionts of corals and clams, 149.
Peridinium, bioluminescence of, 279.
PETERSON, M. K., AND J. BUCK. Light organ fine structure in certain Asiatic fireflies, 335.
Pharmacology of tunicate heart pacemaker, 174.
Pheretima, melting temperature of collagen of, 223.
Pheromone, possible role of in reproductive behavior of Libinia, 273.
Photocyte granules in fireflies, fine structure of, 335.
Photomechanical changes in bluefish retina, 530.
Photosynthetic pigments of symbiotic dinoflagellates, 149.
Photuris, light-inhibition of flashing in, 476.
Physalia, fine structure of gas-secreting tissue of, 486.
Physiological condition of Physalia, as correlated with ultrastructure of gas-secreting tissue, 486.
Pigment composition of algae in coral, 141.
  migration within Uca melanophores, 438.
Pigmentary-effector isohormones in crustaceans, 425.
Pigments, photosynthetic, of symbiotic dinoflagellates, 149.
Pneumadena of Physalia, fine structure of, 486.
Pocillopora, photosynthetic pigments of symbionts of, 149.
PODOLSKY, R. J. See G. B. CALLEJA, 416.
Poikilotherms, temperature relationships of, as correlated with melting temperatures of collagen, 223.
Polarotaxis in copepods, 239, 252.
Polinices, pedal expansion in, 548, 563.
Polychaete, annelid, development of commensal of, 111.
Polyclad, predation of on Corella, 296.
Polyonyx, larval development of, 111.
Polypeptide chain assembly in Opsanus, rate of, 422.
Polyphemus silkmoth, effect of juvenile hormone on synthesis and accumulation of blood protein of, 466.
Pomatomus, rhythmic movement of cones in retina of, 530.
Populations of dinoflagellates, variations in bioluminescence of, 279.
Portuguese man-of-war, fine structure of gas-secreting tissue of, 486.
POSTLETHWAIT, J. H., AND H. A. SCHNEIDERMAN. Effects of an ecdysone on growth and cuticle formation of Drosophila imaginal discs cultured in vivo, 431.
Potentiality of RNA-treated chick embryo, 200.
POTSWALD, H. E. The biology of fertilization and brood protection in Spirorbis, 208.
Predation on Corella, 296.
Predatory behavior of copepods, role of polarotaxis in, 252.
Pressure, effects of on mechanical properties of sea urchin egg membrane, 442.
  -temperature study of Actinosphaerium axopodia, 429.
Primitive streak of chick embryo, development of after liver-RNA treatment, 200.
Proboscidactyla, colony growth and pattern in, 96.
Properties, pharmacological, of tunicate heart pacemaker, 174.
Protein from Arbacia eggs, 193.
  in blood of silkmoth, effect of juvenile hormone on synthesis and accumulation of, 466.
  of connective tissue, melting temperature of, 223.
  synthesis, role of enzymes in control of, 406.
    in dogfish white blood cells, 415.
    in toadfish liver, 130.
Proteins in Xenopus egg jelly, 501.
Protozoan endocommensals of echinoids, 514.

Pteroptyx, fine structure of light organ of, 335.
Pupae of silkmoth, effect of juvenile hormone on blood proteins of, 466.
Puparium formation, role of ecdysone in, 322.
Pyrophanes, fine structure of light organ of, 335.

R

RNA, transfer, specificity in bonding of amino acids to, 181.
  synthesis in toadfish liver, 130.
  -treated chick embryo, development of, 200.
Radiochromium as label for teleost red cells, 105.
Raja embryos, urea and TMAO levels in, 537.
Rana, effects of hydrocortisone on blood of, 92.
Rao, K. R., and M. Fingerman. A comparative analysis of control of white chromatophores in Uca, 432.
Rao, K. R., and M. Fingerman. Local variations of pigmentary patterns and chromatophoric response in the fiddler crab, Uca, 408.
Rate of growth of Loligo, 366.
Read, C. P. See C. Arme, 80.
Read, L. J. Urea and trimethylamine oxide levels in elasmobranch embryos, 537.
Receptor and generator potentials in skate ampullae of Lorenzini, 430.
Red cell morphometrics of teleost, 105.
Reducing substances in blood of crabs, 574.
Regenerating tissues in cockroach, 520.
Regeneration of hydranths in Tubularia, effects of actinomycin-D on, 436.
  of limbs in adult amphibians, 410.
Regulation of urea and TMAO in elasmobranch development, 537.
Reinecke, J. P. See E. P. Marks, 520.
Reinisch, C. L., and F. B. Bang. Cell recognition in an echinoderm, Asterias, 432.
Reproduction of brine shrimp, effects of detergent on, 429.
  of tropical sea urchin, in relation to oxygen uptake, 308.
Reproductive bevarior in Libinia, 273.
  cycle of Spisula, 349.
  performance of insects from Biosatellite II, 408.
Respiration of tropical sea urchin, 308.
Responses, sexual, of male Aedes, 445.
Retina of bluefish, movement of cones in, 530.
Revel, J.-P. See D. A. Goodenough, 420.
Reynolds, G. T. See G. B. Calleja, 416; J. G. Morin, 429; E. Swift, 439.
Rhabdom of naupliar eye in copepods, fine structure of, 252.
Rhythm, endogenous, of polarotaxis in copepods, 239.
Rhythmic movement of cones in bluefish retina, 530.
Ribosomal RNA cistrons in single and multinucleolate oocytes, 441.
Rice, R. V., G. F. Elliott and A. Stracher. Electron microscopical studies on "myosin" from molluscan smooth muscle, 433.
Rice, R. V. See A. Stracher, 438.
Rigby, B. J. Temperature relationships of poikilotherms and the melting temperature of molecular collagen, 223.
Roberts, J. L. Activity levels and cardioregulation of the northern sea robin Prionotus, 433.
Ropes, J. W. Reproductive cycle of the surf clam Spisula in offshore New Jersey, 349.
Russell-Hunter, W. D., and M. L. Apley. Pedal expansion in the naticid snails. II, 563.
Russell-Hunter, W. D., and M. Russell-Hunter. Pedal expansion in the naticid snails. I, 548.
Rustad, R. C. Do ploidy and cytoplasmic volume influence cleavage rate of sea urchin eggs, 434.

S

Sabellid commensal, colony growth of, 96.
Salamander, migratory orientation of, 230.
Sarcophaga, ecdysone secretion and action in, 322.
Schmeer, Sr. A. C. Mercenene clam extract: effects of on cell population kinetics, 434.
Schneiderman, H. A., and A. Krishnakumaran. The control of molting in arthropods by steroid hormones, 435.
Schneiderman, H. A. See M. Blumenfeld, 466; J. H. Postlethwait, 431.
Schopf, T. J. M., and D. F. Travis. Skeletal wall structure of a calcified bryozoan, 436.
Sea urchin, feeding and oxygen uptake of, 308.
  eggs, lipoprotein from, 193.
Sea urchins, Caribbean, endocommensal ciliates of, 514.
Seasonal changes in gonads of surf clams, 349.
  differences in abundance of Corella, 296.
Secreting tissue in Physalia, fine structure of, 486.
Secretion of ecdysone in Sarcophaga, 322.
Segmentation of milliped larvae, effects of diet on, 454.
Seldin, E. B. See G. Wald, 441.

Self-fertilization in Spirorbis, 208.
Semi-starvation, effects of on growth of millipeds, 454.
Sensory nerve regeneration in cockroach, 520.
Seriatopora, photosynthetic pigments of symbionts of, 149.
Setae of discinid larva, 263.
Settling of Corella, 296.
Sex-limited blood protein in silkmoth, effect of juvenile hormone on, 466.
Sexual and asexual reproduction in Pygospio, 410.
  differences in reproductive behavior of spider crabs, 273.
  responses of male Aedes, 445.
Shell of discinid larva, 263.
Shin, D. See W. S. Vincent, 441.
Shoop, C. R. Migratory orientation of Ambystoma, 230.
Sialic acid in Xenopus egg jelly, 501.
Silkmoth, effect of juvenile hormone on synthesis and accumulation of blood protein of, 466.
Singer, I., and J. D. Palmer. The influence of actinomycin-D on distal-hydranth regeneration in Tubularia, 436.
Siphonales algae of coral, pigment of, 141.
Siphonein in algae of coral, 141.
Size distribution of current year class Loligo, 366.
  of sea urchins, in relation to oxygen uptake, 308.
Skate embryos, urea and TMAO levels in, 537.
Snails, naticid, pedal expansion in, 548, 563.
Solitary ascidian, ecology and growth of, 296.
Som, P., and F. B. Bang. The effects of temperature and various biological substances on mucociliary function of intact Nudibranchia, 437.
Sound production by katydid, rapidly contracting muscles used in, 409.
Spawning of Spirorbis, 208.
  of Spisula, 349.
Specificity in bonding of amino acids to tRNA, 181.
Speidel, C. C., and R. H. Cheney. Nicotine-induced alteration of DNA and cell membranes in Arbacia gametes, 437.
Spermatozoan motility in acoel turbellarian, 417, 422.
Spider crab, reproductive behavior in, 273.
Spirorbis, fertilization and brood protection in, 208.
Spisula, reproductive cycle of, 349.
Spotted salamanders, migratory orientation of, 230.
Squid, growth and size distribution of current year class of, 366.
  egg, furrow formation in, 408, 413.
Starfish, amino acid activating enzyme from, 181.
Starmer, C. F. See J. D. Cassidy, 412.
Starvation, effects of on growth of milliped, 454.
Stegomyia, sexual responses of, 445.
Stewart, J. R., and J. P. Green. Pigment migration within fiddler crab melanophores, 438.
Stimulation of Purkinje cells in Mustelus, 418.
Stracher, A., R. V. Rice and G. F. Elliott. ATPase and actin combining properties of "myosin" from molluscan smooth muscle, 438.
Stracher, A. See R. V. Rice, 433.
Strittmatter, P. See L. Laster, 427.
Studies on membrane transport. II, 80.
Stunkard, H. W. Studies on the life-history of Distomum, 439.
Sugars in blood of crabs, 574.
  in Xenopus egg jelly, 501.
Summers, W. C. The growth and size distribution of current year class Loligo, 366.
Surf clam, reproductive cycle of, 349.
Swift, E., and G. T. Reynolds. Localization of bioluminescence in the marine dinoflagellate Pyrocystis using an image intensifier, 439.
Swimming of bluefish, as related to movement of cones in retina, 530.
Symbiont of coral, pigments of, 141.
Symbiotic dinoflagellates, photosynthetic pigments of, 149.
Synthesis of blood protein in silkmoth, effect of juvenile hormone on, 466.
  of protein in toadfish liver, 130.

T

TMAO levels in elasmobranch embryos, 537.
tRNA, specificity in bonding of amino acids to, 181.
Taborikova, H. See J. C. Eccles, 418; N. Tsukahara, 440.
Tactile behavior by regenerating cockroach nerves, 520.
Tadpole blood, effects of hydrocortisone on, 92.
Tapeworm, absorption of lipids by, 80.
Tasaki, I. See A. Watanabe, 442.
Teleost, movement of cones in retina of, 530.
  protein synthesis in liver of, 130.
  red cell morphometrics and volume in, 105.

Telford, M. The identification and measurement of sugars in the blood of three species of Atlantic crabs, 574.

Temes, S. See G. C. Candelas, 415.

Temperature-acclimated toadfish, protein synthesis in liver of, 130.
  in relation to oxygen uptake of sea urchins, 308.
  relationships of molecular collagen, 223.
  in relation to stability of ecdysone, 322.
  role of in larval development of Polyonyx, 111.
  water, in relation to Spisula reproductive cycle, 349.

Thalassopsammon, extraction of from intertidal and subtidal sands, 443.

Thigmotropic behavior by cockroach regenerating nerves, 520.

Thrombocytes of Tilapia, 105.

Tilapia, red cell morphometrics and volume in, 105.

Tisbe, polarotactic behavior of, 252.

Tissues, regenerating, of cockroach, 520.

Toadfish, amino acid activating enzyme from, 181.
  liver, protein synthesis in, 130.

Tracheal end organs and tracheoles, fine structure of, in fireflies, 335.

Transfer RNA, specificity in bonding of amino acids to, 181.

Transpeptidating enzymes, continuous rate assay for, 430.

Transport, membrane, 80.

Travis, D. F. See T. J. M. Schopf, 436.

Trehalase, new source of, 419.

Tridacna, photosynthetic pigments of symbiont of, 149.

Trimethylamine oxide levels in elasmobranch embryos, 537.

Trinkaus, J. P. See M. V. L. Bennett, 415.

Trinkle, M. S. See J. Case, 476.

Tripneustes, endocommensal ciliates of, 514.

Troll, W., A. Grossman and S. Chasis. Fertilization-induced increase of trypsin-like activity in sea urchin "membrane" during embryogenesis, 440.

Tropical sea urchin, feeding and oxygen uptake of, 308.

True, R. S. See M. Fingerman, 418.

Tsukahara, N., H. Taborikova and J. C. Eccles. The responses of granule cells in the cerebellum of Mustelus, 440.

Tsukahara, N. See J. C. Eccles, 418.

Tubocurarine, effects of on tunicate heart pacemaker, 174.

Tunicate heart cells, pacemaker properties of, 166.

Tunicate heart pacemaker, pharmacology of, 174.

Tuttle, J. See S. Lerman, 428.

U

Ultrastructural basis for polarotaxis in copepods, 252.

Ultrastructure of firefly light organ, 335.
  of gas-secreting tissue of Physalia, 486.

Umminger, B. L. Polarotaxis in copepods. I, 239; II, 252.

Uptake of fatty acids by cestode, 80.
  of oxygen by Eucidaris, 308.

Urea levels in elasmobranch embryos, 537.

V

Van Holde, K. E., and H. A. DePhillips, Jr. Oxygen-linked dissociation of hemocyanin. II, 441.

Van Holde, K. E. See H. A. DePhillips, Jr., 417.

Vertical migration of copepods, in relation to polarotaxis, 239.

Vincent, W. S., H. O. Halvorson, H. R. Chen and D. Shin. Ribosomal RNA cistrons in single and multinucleolate oocytes, 441.

Virgin female mosquitoes, sexual responses of, 445.

Vitellogenesis in Artemia, quantitative autoradiography of, 412.

Volume, red cell, in teleost, 105.

W

Wald, G., and E. B. Seldin. The electroretinogram and spectral sensitivity of the common scallop, 441.

Watanabe, A., I. Tasaki and L. Carnay. Optical changes associated with nerve excitation and membrane polarization, 442.

Water content in elasmobranch embryos, 537.
  -sinus system in naticid snails, 548, 563.

Waxman, S. G. See M. E. Kriebel, 426.

Williams, C. M. See T. Ohtaki, 322.

Wolpert, L., D. Marsland and M. Hirshfield. The effect of high hydrostatic pressure on the mechanical properties of the membrane of the sea urchin egg, 442.

Worm collagen melting temperature, 223.
  commensal, colony growth of, 96.

X

Xanthophyll in algae of coral, 141.
  in symbionts of corals and clams, 149.

Xenia, photosynthetic pigments of symbiont of, 149.

Xenopus egg, cytochemistry of jelly layers of, 501.

Y

Yeast, amino acid activating enzymes from, 181.

Yolk protein of silkmoth, effect of juvenile hormone on synthesis and accumulation of, 466.

YONGE, C. M. Form and habit in species of Malleus, 378.

Z

ZIGMAN, S., AND S. J. BAGLEY. The effect of light on the dogfish eye, 443.

ZINN, D. J. Comments on the extraction of Thalassopsammon from intertidal and subtidal sands, 443.

ZIGMAN, S. Sulfonation of rat lens proteins, 406.

Zooxanthellae, symbiotic, photosynthetic pigments of, 149.

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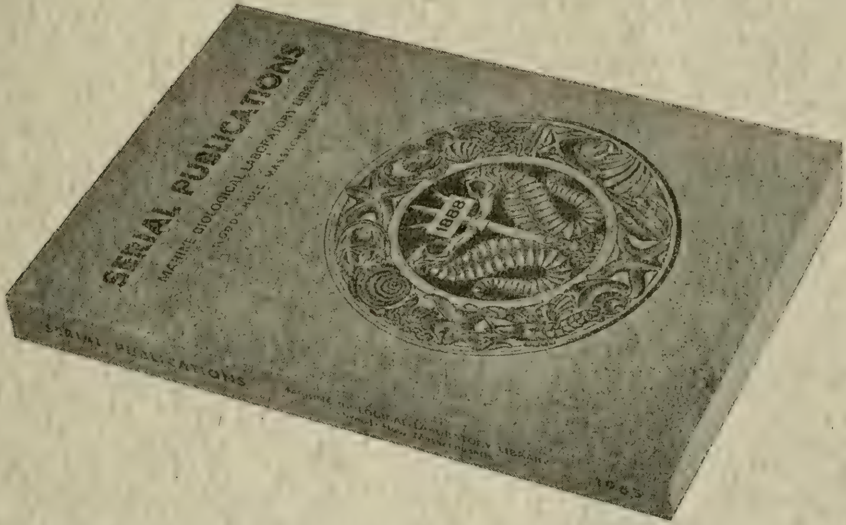
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CONTENTS

Page

AKEY, DAVID HAROLD, AND JACK COLVARD JONES
Sexual responses of adult male Aedes aegypti using the forced-copulation technique 445

BERNS, MICHAEL W., AND WILLIAM T. KEETON
Effects of semi-starvation on growth and morphogenesis during the larval stages of a common milliped, Narceus annularis (Raf.) 454

BLUMENFELD, MARTIN, AND HOWARD A. SCHNEIDERMAN
Effect of juvenile hormone on the synthesis and accumulation of a sex-limited blood protein in the Polyphemus silkmoth 466

CASE, JAMES, AND M. S. TRINKLE
Light-inhibition of flashing in the firefly Photuris missouriensis 476

COPELAND, D. EUGENE
Fine structure of the carbon monoxide secreting tissue in the float of Portuguese man-of-war (Physalia physalis L.) 486

FREEMAN, SALLIE B.
A study of the jelly envelopes surrounding the egg of the amphibian, Xenopus laevis 501

JONES, IRA, AND THOMAS E. ROGERS
Studies on the endocommensal ciliate fauna of Caribbean sea urchins 514

MARKS, E. P., J. P. REINECKE AND R. A. LEOPOLD
Regenerating tissues from the cockroach, Leucophaea maderae: Nerve regeneration in vitro 520

OLLA, BORI L., AND WARREN W. MARCHIONI
Rhythmic movements of cones in the retina of bluefish, Pomatomus saltatrix, held in constant darkness 530

READ, LAWRENCE J.
Urea and trimethylamine oxide levels in elasmobranch embryos 537

RUSSELL-HUNTER, W. D., AND MYRA RUSSELL-HUNTER
Pedal expansion in the naticid snails. I. Introduction and weighing experiments 548

RUSSELL-HUNTER, W. D., AND MARTYN L. APLEY
Pedal expansion in the naticid snails. II. Labelling experiments using inulin 563

TELFORD, MALCOLM
The identification and measurement of sugars in the blood of three species of Atlantic crabs 574

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